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15. SUBJECT TERMS
spinal cord injury, weight drop injury, P2X7 receptors, locomotor function, experimental model, rat, mouse, small molecule experimental treatment, microglia, astrocytes

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Table of Contents

Development of a Small Molecule P2X7R Antagonist as a Treatment for Acute Spinal Cord Injury

Principal Investigator: Maiken Nedergaard, MD/PhD

Abstract/Introduction

This project is based on the premise that secondary damage following traumatic spinal cord can be limited by acute administration of P2X7 receptor antagonists. The objectives of the proposal were to screen P2X7 receptor antagonists in a weight drop model of spinal cord injury, define the clinical indications for administering the P2X7 receptor antagonist in various models of SCI, and obtain data for safety and toxicity data to support an IND application necessary to conduct a clinical trial. We have thus far made good progress and have validated that two P2X7 receptor antagonists, BBG and A-740003 have neuroprotective benefits in the setting of spinal cord injury in two species: rats and mice. Two other receptor antagonists (KN-62 and MRS2159) did not provide clinical benefits. An interesting discovery is that BBG possess neuroprotective effects that in part, are mediated by suppressing the endogenous inflammatory response to tissue injury, by P2X7 receptor independent pathways. This observation was based on the analysis of P2X7 receptor knockout mice. We believe that this observation is potentially of great importance because of BBG: The prospect of administering a small drug with no known adverse effects is particularly attractive in the setting of acute traumatic injury because transportation of patients with SCI is a specific concern.

Body (Progress report)

In Year 2 of this project we focused on Aims 1, 2, and 4.

Aim 1

We established in Year 1 of the project that the P2X7 receptor antagonists, MRS2159 and KN-62, in the maximal dose, tolerated MRS2159 (30-50 mg/kg), and KN-62 (20-40 mg/kg). These agents did not afford significant neuroprotection compared to BBG (10 mg/kg). Adult female Sprague–Dawley rats (220–250g) were anesthetized with i.p. injections of a mixture containing 8 mg/kg ketamine and 10 mg/kg xylazine. For surgery, a midline incision was made on the back region and a laminectomy was performed aseptically at the T11-T12 level. Before SCI, a catheter was placed in the left femoral vein after carefully separating nerves and blood vessels. Immediately afterward, the exposed dorsal surface of the cord was subjected to a 10g weight-drop impact from a height of 12.5 mm. Vehicle or MRS2159, KN-62 or BBG were given intravenously 10–15 min after the weight drop. BBG served as our positive control, because this P2X7 receptor antagonist previously has been shown to improve functional recovery and reduced lesion volume in rats exposed to traumatic weight drop injury of the spinal cord (Peng et al., 2009). We reported in the last progress report that

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MRS2159 (50 mg/kg) and KN-62 (40 mg/kg) did not provide a significant functional improvement in the maximal doses tolerated by the rats, whereas A-740003 (40 mg/kg) significantly improved locomotor function in rats exposed to traumatic spinal cord injury, compared to control vehicle-treated littermates. Based on these observations, we decided to not pursue additional work on MRS2159 and KN-62, as these antagonists had no effects on the severity of SCI in rats. Rather, in Year 2 of the project, we have focused on continuing the studies proposed in Aim 1 and constructed a dose-response of the neuroprotective effect of BBG and A-740003 in a mouse model of spinal cord injury.

We repeated the analysis of BBG and confirmed that BBG consistently improved functional recovery and reduced the lesion volume in mice with SCI. It was needed to repeat the analysis of the neuroprotective effects of BBG, because (1) it was critical to establish whether BBG has neuroprotective effects in another species (mice), and (2) we could test whether BBG acts solely though inhibition of P2X7 receptors, or alternatively, if BBG protects against secondary injury in P2X7 receptor independent pathways by administering BBG to P2X7 receptor knockout mice (KO) and compare outcome to P2X7 receptor KO mice exposed to the same injury and receiving vehicle (PBS). The analysis first included an analysis of the effect of BBG in littermate, wild type controls, in addition to P2X7 receptor KO mice. These studies were necessary as variations in the gene expression of inbreed transgenic mice line significantly can affect the SCI outcome. We confirmed that BBG, at a dose of 2, 10, and 50 mg/kg, significantly improved functional outcome (Fig. 1). The higher doses of BBG (10 and 50 mg/kg) also reduced the lesion volume in wild type controls to the P2X7R KO mice (Fig. 2). The analysis was next extended to include P2X7 receptor KO mice. Surprisingly, BBG protected, albeit to a lesser degree, P2X7 receptor KO mice against secondary injury: Functional recovery was suppressed in P2X7 receptor KO mice (at 10 and 50 mg/kg) compared to their litter mate controls receiving BBG (Fig. 1). Similarly, the traumatic lesion exhibited an insignificant trend toward a reduction in P2X7 receptor KO mice receiving 10 and 50 mg/kg BBG, compared to their wild type littermates (Fig. 2). Combined, this analysis indicates that BBG have neuroprotective targets other than the inhibition of P2X7 receptors.

An extensive immunohistochemical analysis showed that P2X7 receptor KO mice exhibit less inflammatory changes when evaluated 4 days after spinal cord injury in comparison to littermate controls. The analysis included immunolabeling against Iba1 and CD68 (microglial cells), GFAP (astrocytes), MPO (neutrophils) and CD8 (T cells).
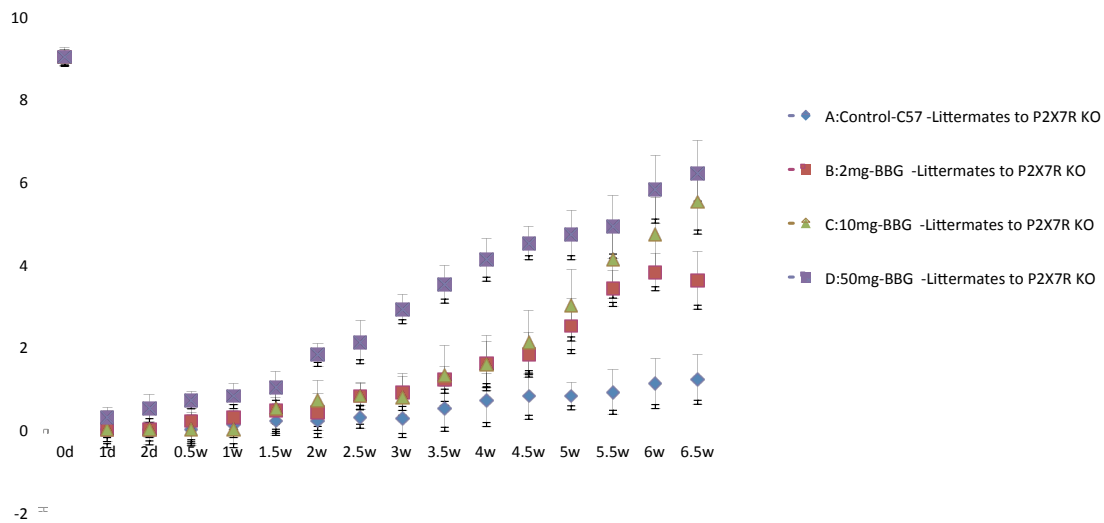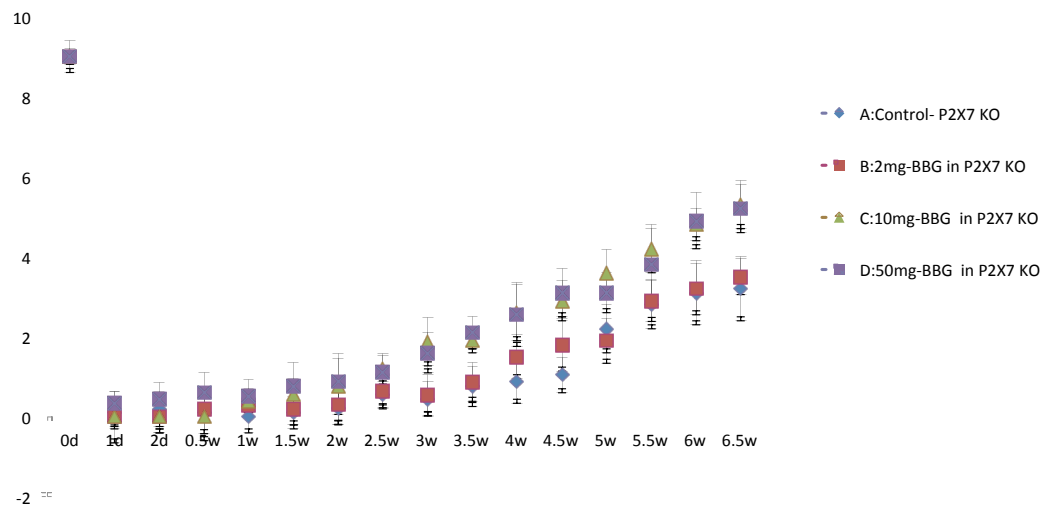
A

- ◆ A:Control-C57 -Littermates to P2X7R KO
- ■ B:2mg-BBG -Littermates to P2X7R KO
- ▲ C:10mg-BBG -Littermates to P2X7R KO
- ■ D:50mg-BBG -Littermates to P2X7R KO

B

- ◆ A:Control- P2X7 KO
- ■ B:2mg-BBG in P2X7 KO
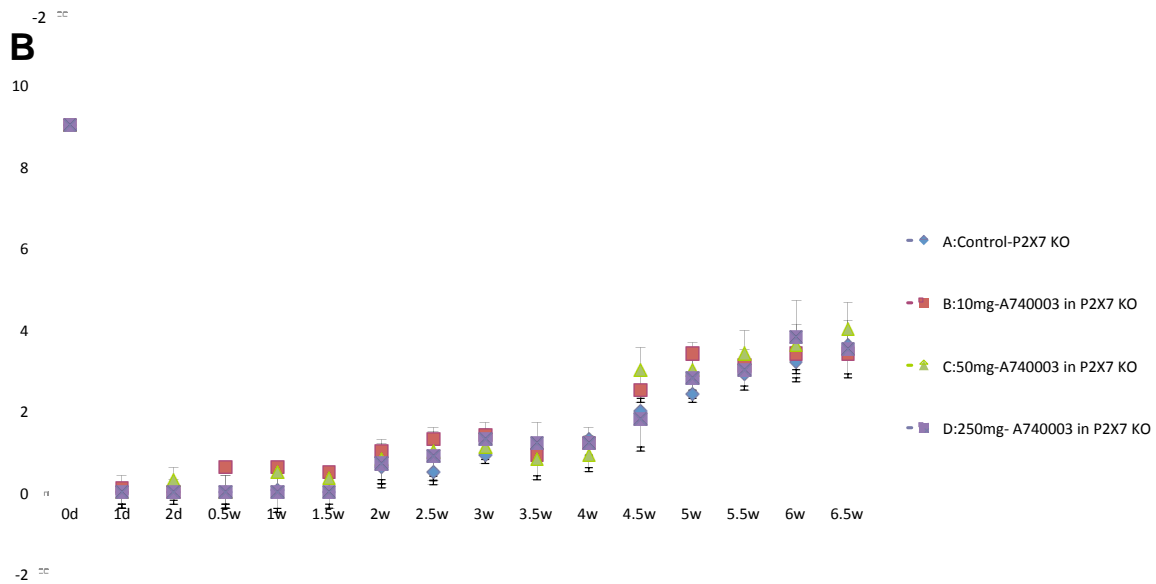- ▲ C:10mg-BBG in P2X7 KO
- ■ D:50mg-BBG in P2X7 KO

Fig. 1. Effect of BBG on locomotor recovery after spinal cord injury on P2X7 receptor KO mice and their wildtype litter mates. (A) All doses of BBG (2, 10, 50 mg/kg) improved functional recovery in wild type mice (littermate to P2X7 receptor KO mice. (**B**) The two higher doses of BBG also improve functional recovery in P2X7 receptor KO mice. Female mice of 8–10 weeks of age (15–25g) were anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg,i.p.). A laminectomy of the dorsal portion of T11 was performed, and the vertebral column was held with fine clamps at the T10 and T12 levels. The exposed dorsal surface of the spinal cord was subjected to a drop of a 3g weight with flat tip (diameter 0.5 mm) from a height of 12.5 mm (modified NYU impactor). Lesions were lateral or on the midline. The Basso Mouse Scale for Locomotion (BMS) rating scale, from 0 (no ankle movement) to 9 (normal gait), was used for evaluating hindlimb movement. The mice were blindly evaluated for 6.5 weeks.

A

Littermate to P2X7R- KO
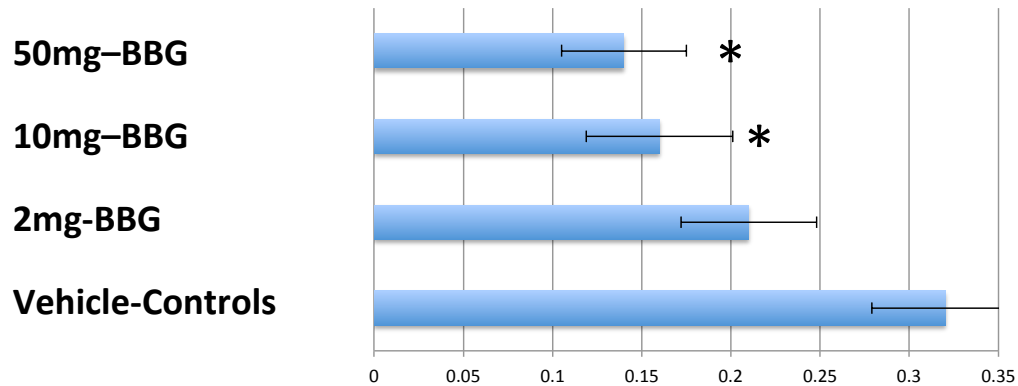
P2X7R- KO
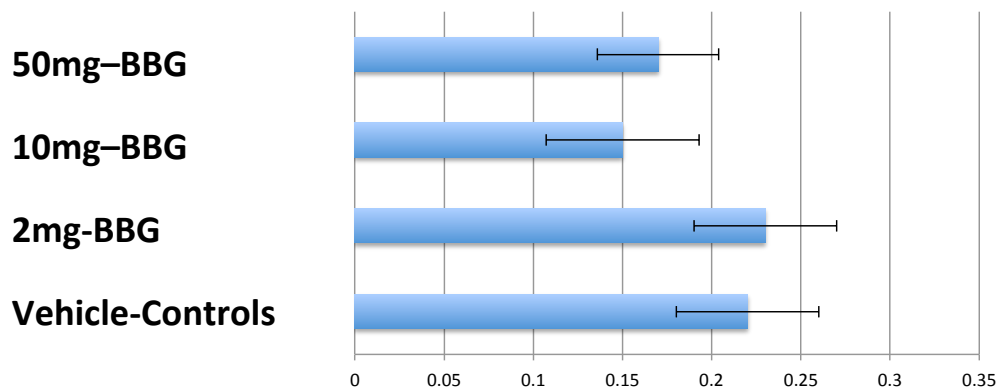
B
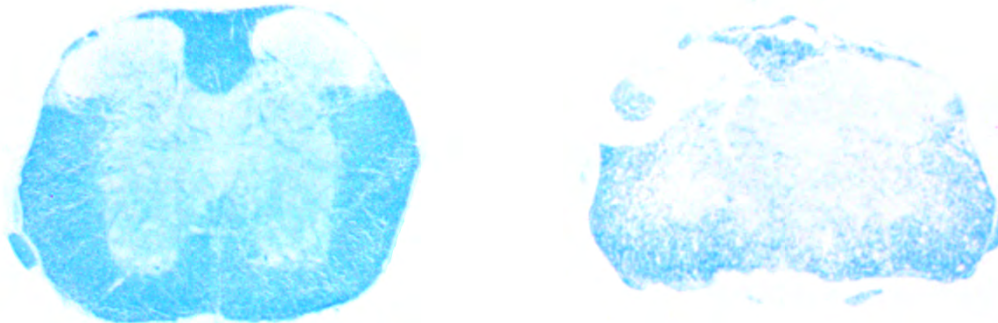
Fig. 2. Comparison of the volume of the traumatic lesion 6.5 weeks after SCI in P2X7 receptor KO and littermate controls exposed to SCI and treated with 0, 2, 10, or 50 mg/kg BBG. (A) All the animals included in the analysis of locomotor recovery after SCI (Fig. 1), were included in the histological analysis. **(B)** Lesion volumes were determined on serial sections stained with Luxol Fast Blue and cresyl violet. Representative examples of non-injured (right panel) and injured spinal cord (left panel) is shown.
(n = 16, +, p < 0.05 ANOVA, Bonferroni).

We next continued the evaluation of A-740003, a highly selective P2X7 receptor antagonist. This agent was developed by Dr. Surprenant at Manchester University, in an effort to develop an experimental therapeutic for arthritis pain. We collected encouraging data that suggested that A-740003 indeed improved functional recovery and reduced the lesion volume in wildtype littermates (Fig. 3). This observation is important in that A-740003 is more selective than BBG in antagonizing P2X7 receptors. Indeed, administering A-740003 to P2X7 receptor KO mice did not improve their function outcome or tissue injury. Thus, this analysis provides compelling arguments for P2X7 receptors as a therapeutic target following traumatic spinal cord injury.

A
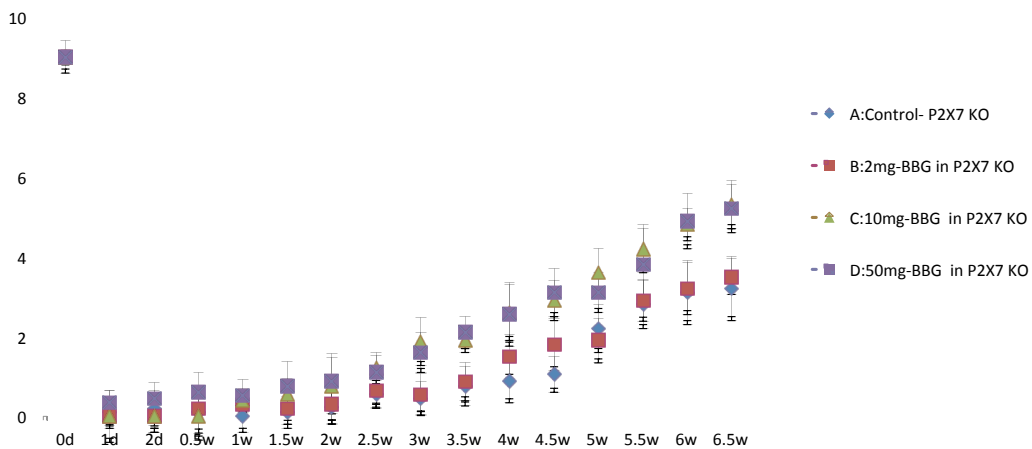
B

Fig. 3. Effect of A740003 on locomotor recovery after spinal cord injury in P2X7 receptor KO mice and their wildtype litter mates. The two higher doses of A740003 (10, 50 mg/kg) improved functional recovery in wild type mice. However, no effect of A740003 was noted in P2X7 receptor KO mice. These mice exhibit consistently improved locomotor recovery compared to their wildtype

littermates (compare figs 1 and 3) consistent with the notion that P2X7 receptors contribute to the innate inflammatory response to injury.

We next analyzed the lesion volume after traumatic spinal cord injury in P2X7 receptor KO and their littermates receiving treatment with A740003 (2, 10, or 50 mg/kg) or vehicle (**Fig. 4**). The analysis showed that A740003 significantly reduced the lesion size in wildtype mice (littermate to P2X7 receptor KO mice) compared to the lower dosis of A740003 or vehicle (PBS) controls (Fig. 4). No neuroprotective effect was noted on the lesion volume in P2X7 receptor KO mice exposed to SCI. This observation is consistent with the observation that A740003 consistently did not improve functional recovery of P2X7 receptor KO mice and that A740003 is highly specific P2X7 receptor antagonist.
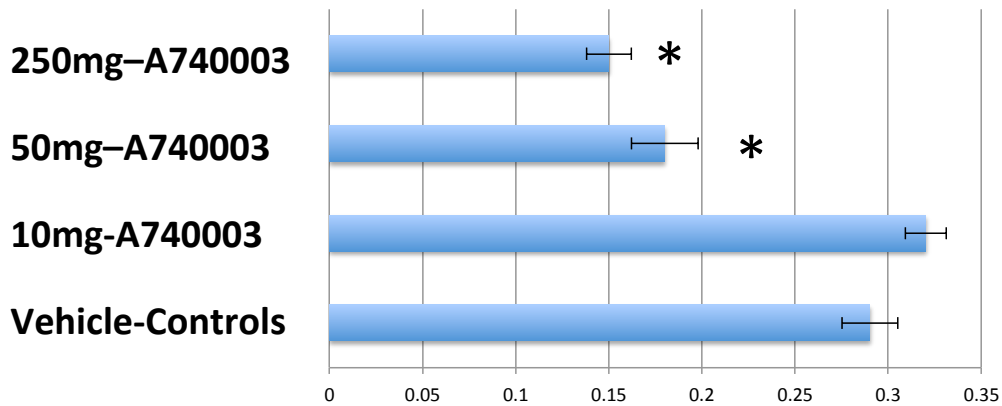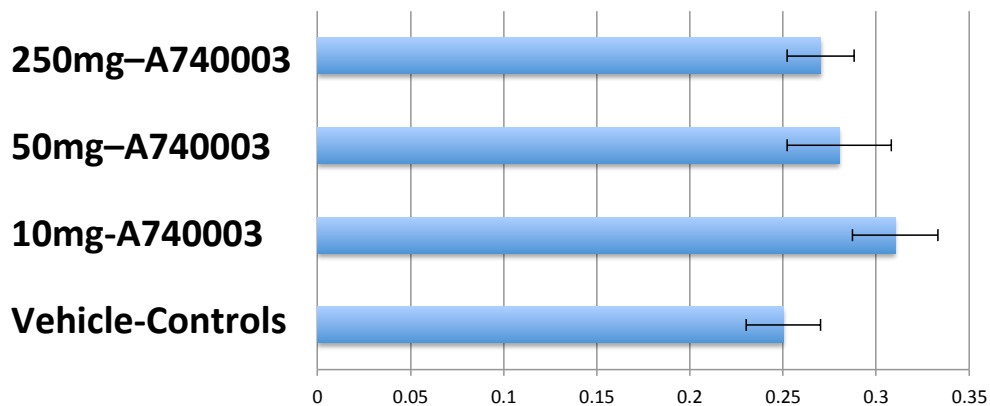
Fig. 4. Comparison of the volume of the traumatic lesion 6.5 weeks after SCI in P2X7 receptor KO and littermate controls exposed to SCI treated with A740003.
(n = 16, +, p < 0.05 ANOVA, Bonferroni).

Based on the observation that BBG afford neuroprotection in P2X7 receptor KO mice, we have analyzed the direct effect of BBG on P2X7 receptor channel activation using electrophysiological approaches. The rational was that it is key to establish whether BBG (and other P2X7 receptor antagonists) indeed inhibit ATP-induced increases in P2X7 receptor currents. This analysis showed that both BBG and A740003 inhibited P2X7 receptor channels with a high affinity. Interestingly, the potency by which MRS2159 and KN-62 antagonized the ATP-induced P2X7 receptor channel current was low, possibly explaining why these two agents did not afford protection. The electrophysiological analysis was useful as it provided us with a tool to screen the efficacy of P2X7 receptor antagonists, prior to initiating the labor intensive preclinical studies of their neuroprotective potential, to reduce secondary injury following spinal cord injury (including surgery, nursing care, administration of the drugs, behavioral assessment, perfusion fixation, tissue processing and staining, 2-photon imaging and processing, as well as image analysis).

In addition to these studies, we have completed an extensive immunohistochemical analysis of the effects of P2X7 receptor antagonists on the inflammatory response to SCI. In these studies, the spinal cord was harvested four days after the traumatic injury. The observations clearly showed that BBG and A740003 in a dose-dependent manner reduce the innate immune response to tissue injury.
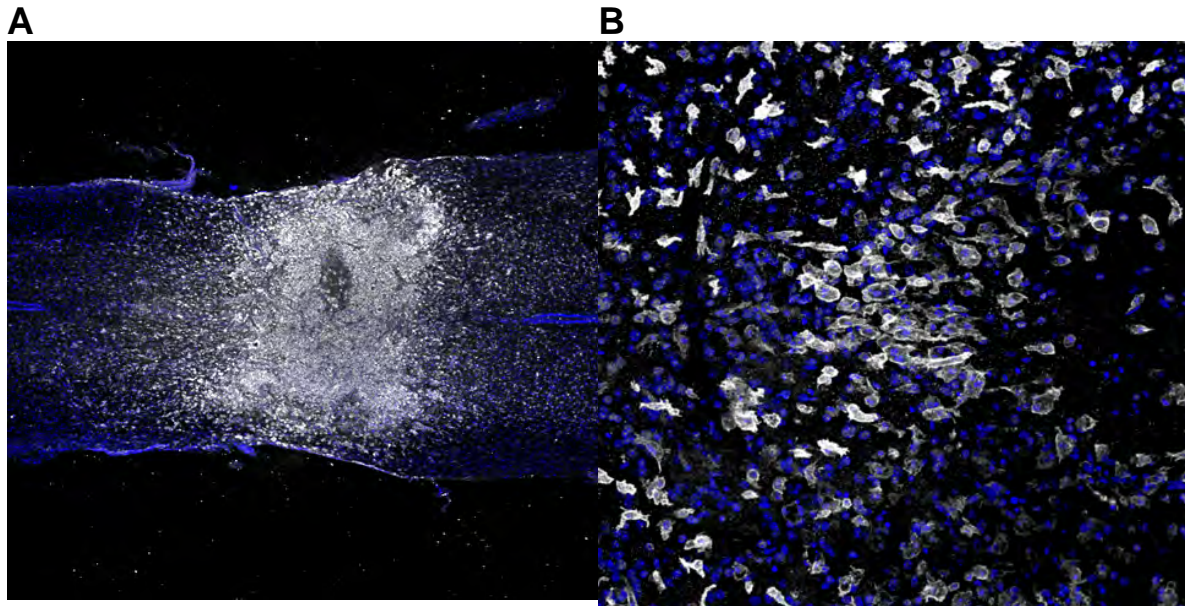

Fig. 5. Microglia cells densely infiltrate spinal cord 4 days after traumatic injury. (**A**) Microglial cells are labeled with iba1 (white) whereas nuclei are stained with DAPI. Low power of whole spinal cord mounts. (**B**) High power depicts the activated state of the microglial cells with retracted processes in the peri-traumatic region.
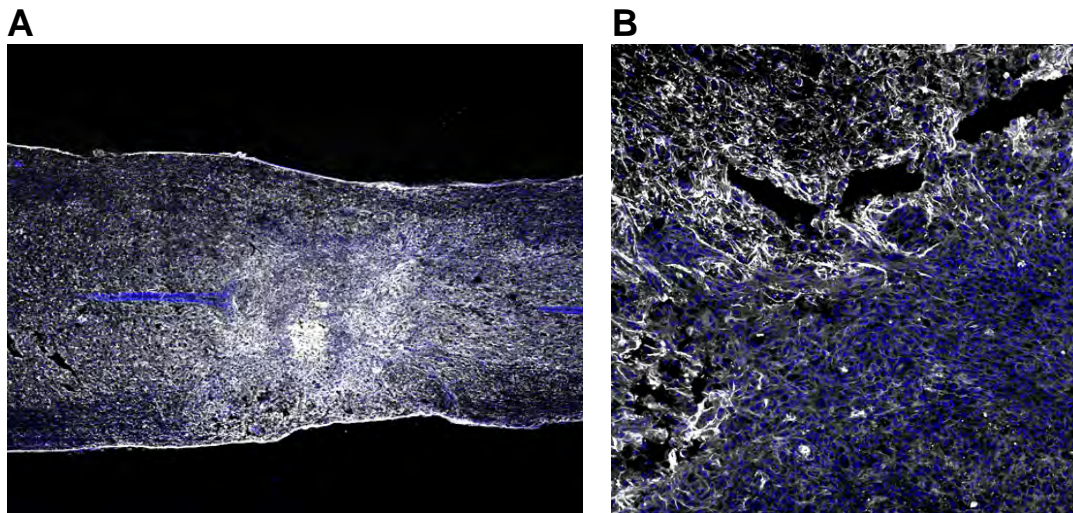
Fig. 6. Reactive astrogliosis in spinal cord 4 days after traumatic injury. (**A**) Reactive gliosis depicted by labeling against GFAP (white) whereas nuclei are stained with DAPI. Low power of a longitudinal section of the spinal cord. (**B**) High power depicts the reactive glial cells forming a scar tissue.
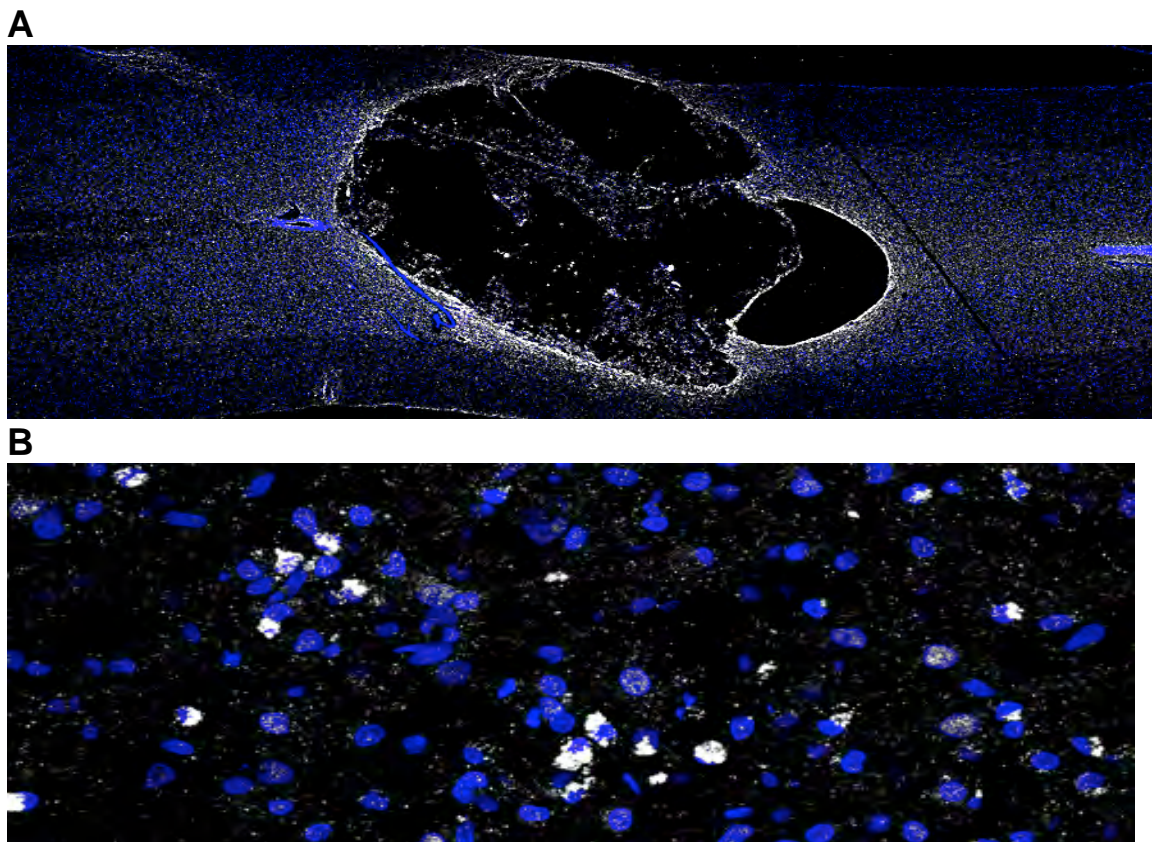

Fig. 7. Neutrophil invasion in spinal cord 4 days after traumatic injury. (**A**) Low power confocal image show the dense infiltration of neutrophils labeled with

a MPO antibody 4 days after SCI (white). Nuclei are counter stained with DAPI. (**B**) High power image of MPO labeled neutrophils in the immediate surroundings of the traumatic lesion.

We have also initiated the experiment involving spinal hemisections and dorsal root avulsion. Both of these procedures induced consistent injury and the models will be fully validated to use when the optimal P2X7 receptor antagonist has been identified.

Future plans:

We plan to screen the effect of the P2X7 receptor antagonist AZ10606120 and MRS 2159. In fact, this analysis has already been initiated and both agents show promising effect.

KEY RESEARCH ACCOMPLISHMENTS:

- Validated that the P2X7 receptor antagonist BBG improves functional recovery and reduces the severity of tissue injury after SCI in two species: rats and mice
- Defined the optimal neuroprotective doses of BBG and A740003
- Defined the therapeutic window of BBG and A740003 in rats and mice
- Established that BBG suppress inflammation in both a P2X7 receptor dependent and independent pathway
- Documented that KN-62 and MRS2159 are not neuroprotective in the setting of SCI
-

REPORTABLE OUTCOMES:

Published reports

1. Huang C, Han X, Li X, Lam E, Peng W, Lou N, Torres A, Yang M,Garre JM, Tian GF, Bennett MV, Nedergaard M, Takano T. (2012) Critical role of connexin 43 in secondary expansion of traumatic spinal cord injury. *J. Neurosci.* 7;32 (10):3333-8.

2. Lovatt D, Xu Q, Liu W, Takano T, Smith NA, Schnermann J, Tieu K, Nedergaard M. (2012) Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity. *PNAS* 109(16):6265-70.

3. Verkhratsky A, Sofroniew MV, Messing A, deLanerolle NC, Rempe D, Rodriguez JJ, Nedergaard M (2011). Neurological diseases as primary gliopathies: a reassessment of neurocentrism. ASN NEURO 4(3):art:e00082.

4. Thrane AS, Rappold PM, Fujita T, Torres A, Bekar LK, Takano T, Peng W, Wang F, Thrane VR, Enger R, Haj-Yasein NN, Skare Ø, Holen T, Klungland A,

Ottersen OP, Nedergaard M, Nagelhus EA. (2011) Critical role of aquaporin-4 (AQP4) in astrocytic Ca2+ signaling events elicited by cerebral edema. *Proc Natl Acad Sci U.S.A.* 108(2):846-51.

5. Cotrina ML, Nedergaard M. (2012) Brain connexins in demyelinating diseases: Therapeutic potential of glial targets. *Brain Res.* Available online 9 July 2012.

6. Chen MJ, Kress B, Han X, Moll K, Peng W, Ji RR, Nedergaard M. (2012). Astrocytic CX43 hemichannels and gap junctions play a crucial role in development of chronic neuropathic pain following spinal cord injury. *Glia* 60(11): 1660-70.

7. Peng W, Takano, T, Han, X, Nedergaard (2012). Comparison of the neuroprotective effect of the P2X7 receptor antagonist, BBG, in rat and mice following traumatic spinal cord injury (in submission).

Two newly sponsored NIH RO1 awards will continue this work:

1. **R01NS075177**: ATP as the instigator of inflammatory responses to spinal cord injury (PI Nedergaard): 2/1/12-1/31/17: 5 years;

2. **R01DE022743**: Hemichannels, astrocytic release, and neuropathic pain (PIs Nedergaard, Ji): 9/1/12-8/31/17: 5 years

CONCLUSION: Several observations show that ATP release and the activation of P2X7 receptors drive the innate inflammatory response to tissue injury. P2X7 receptors are low affinity ATP receptors, only activated in the setting of large increases in the extracellular concentration of ATP. Our earlier studies have shown ATP is released in large quantities from peri-traumatic areas for up to 8 hours after the traumatic event. P2X7R activation activates and coordinates a number of downstream signaling events, including the release of pro-inflammatory cytokines. Suppressing activation of P2X7 receptors - the very initial steps in the innate inflammatory response to spinal cord injury- could be more efficient than targeting complex downstream inflammatory pathways. The objectives of the proposal were to: **Aim 1** screen P2X7 receptor antagonists for their neuroprotective activities in a weight drop model of spinal cord injury in rodents; **Aim 2** define the clinical indications for administering the P2X7 receptor antagonist in various models of SCI in rats; **Aim 3** use a FDA accredited commercial laboratory to obtain GLP rat and rabbit safety and toxicity data, to support an IND application necessary to conduct a collapse phase 1 + 2 clinical trial after completion of the proposed studies; and **Aim 4** define the cellular target for P2X7 receptor blockade. The development of a systemic treatment that antagonize secondary damage after traumatic spinal cord injury and which could be given to soldiers and others following injury on the battlefield has the potential to dramatically improve the outcomes of these injuries, improving the quality of life and reducing the health care needs for thousands of individuals each year.

The prospect of administering a small molecule agent with no known adverse effects is particularly attractive in the setting of acute traumatic injury because transportation of patients with SCI is a specific concern and therefore can be delayed. We have so far made good progress on the project and have validated that two P2X7 receptor antagonists, BBG and A-740003 have neuroprotective benefits in the setting of spinal cord injury in two species, rats and mice. However, progress was slowed by the discovery that the neuroprotective effect of the P2X7 receptor antagonist, BBG, in part is mediated by P2X7 receptor independent pathways. This observation was based on an in-depth analysis of the effect of the P2X7 receptor antagonists in P2X7 receptor knockout mice. This observation has led to a promising analysis of the efficacy by which BBG suppress the endogenous inflammatory response to tissue injury. We believe that this analysis has great potential impact because reducing post-traumatic tissue swelling, and thereby secondary ischemia and tissue loss, bears great promise for improving functional recovery. The next step will be to define the therapeutic benefits of administration of A-438079 and AZ10606120 after SCI in WT and P2X7 receptor KO mice and expand the analysis of anti-inflammatory action of P2X7 receptor-independent actions of BBG.

REFERENCES: References are listed under 'Reportable Outcomes.'

APPENDICES: 6 published articles

SUPPORTING DATA: Figures are included in main text

Brief Communications

Critical Role of Connexin 43 in Secondary Expansion of Traumatic Spinal Cord Injury

Chunlan Huang,[1]* Xiaoning Han,[1]* Xi Li,[1]* Eric Lam,[1] Weiguo Peng,[1] Nanhong Lou,[1] Arnulfo Torres,[1] Meixiang Yang,[1] Juan Mauricio Garre,[2] Guo-Feng Tian,[1] Michael V. L. Bennett,[2] Maiken Nedergaard,[1] and Takahiro Takano[1]

[1]Division of Glial Disease and Therapeutics, Center for Translational Neuromedicine, Department of Neurosurgery, University of Rochester, Rochester, New York 14642, and [2]Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

Spinal cord injury (SCI) is often complicated by secondary injury as a result of the innate inflammatory response to tissue trauma and swelling. Previous studies have shown that excessive ATP release from peritraumatic regions contributes to the inflammatory response to SCI by activation of low-affinity P2X7 receptors. Because connexin hemichannels constitute an important route for astrocytic ATP release, we here evaluated the impact on post-traumatic ATP release of deletion of connexins (Cx30/Cx43) in astrocytes. *In vivo* bioluminescence imaging showed a significant reduction in ATP release after weight-drop injury in mice with deletion of Cx43 compared with Cx43-expressing littermates, both on a Cx30 knockout background. Moreover, astrogliosis and microglia activation were reduced in peritraumatic areas of those mice lacking Cx43; motor recovery was also significantly improved, and the traumatic lesion was smaller. Combined, these observations are consistent with a contribution by astrocytic hemichannels to post-traumatic ATP release that aggravates secondary injury and restrains functional recovery after experimental spinal cord injury. Connexins may thereby constitute a new therapeutic target in spinal cord injury.

Introduction

It is estimated that in the United States alone ∼400,000 individuals are living with spinal cord injury (SCI) with more than 14,000 new cases occurring each year (Sekhon and Fehlings, 2001). Although acute inflammatory response is a defense mechanism aimed at preserving tissue integrity and demarcating the traumatic lesion (Bethea, 2000), an exaggerated response may limit the potential for successful recovery (Popovich and Longbrake, 2008). Tissue swelling, especially within the tight confines of the vertebral canal, can reduce tissue perfusion and cause secondary ischemia. The delayed loss of tissue affects functional recovery in most patients, and no effective treatment options currently exist.

It was shown 50 years ago that injection of ATP in the absence of injury was sufficient to induce acute inflammatory responses. A key observation linking purine signaling to inflammatory mediators was that activation of a purinergic receptor P2RX7 triggers maturation and secretion of IL-1β from microglial cells (Di Virgilio et al., 1999). Although it is recognized that adenine nucleotides (i.e., ATP and its metabolites) are inflammatory mediators, the role of purinergic signaling in spinal cord injury has received relatively little attention (Cotrina and Nedergaard, 2009). We showed previously that spinal cord injury leads to excessive and sustained ATP release in peritraumatic regions and inhibition of P2RX7 reduces inflammatory responses and improves functional recovery.

Previous studies showed that astrocytes release ATP, at least in part, by the opening of connexin43 (Cx43) hemichannels (Cotrina and Nedergaard, 2009). Connexins are a family of proteins with dual channel functions (Bennett et al., 2003). The traditional role is to form gap junctions, which are composed of two docked hemichannels linking the cytosol of two neighboring cells. Gap junctions allow cell-to-cell passage of ions and small molecules, including Ca^{2+}, cAMP, IP_3, ATP, glutamate, and glucose. It has been acknowledged that unopposed hemichannels constitute a pathway for regulated gliotransmitter release (Bennett et al., 2003). Because of their relatively large inner-pore diameter (∼10 Å), open hemichannels facilitate efflux of small cytosolic compounds, and many of these, including ATP and glutamate, will act as transmitters once released (Cotrina et al., 1998; Parpura et al., 2004). Hemichannel openings are normally tightly controlled, because prolonged opening of many hemichannels is incompatible with cellular survival. We sought here to define the role of Cx43 in post-traumatic ATP release and secondary injury after SCI.

Materials and Methods

Spinal cord injury and bioluminescence imaging of extracellular ATP. Knockout Cx30 and floxed Cx43 mice (Cx30$^{-/-}$,Cx43$^{fl/fl}$) and mice expressing Cre under the human GFAP (hGFAP) promoter were obtained from Klaus Willecke's laboratory (University of Bonn, Bonn, Germany) (Theis et al., 2001). Female mice of 8–10 weeks of age (15–25 g)
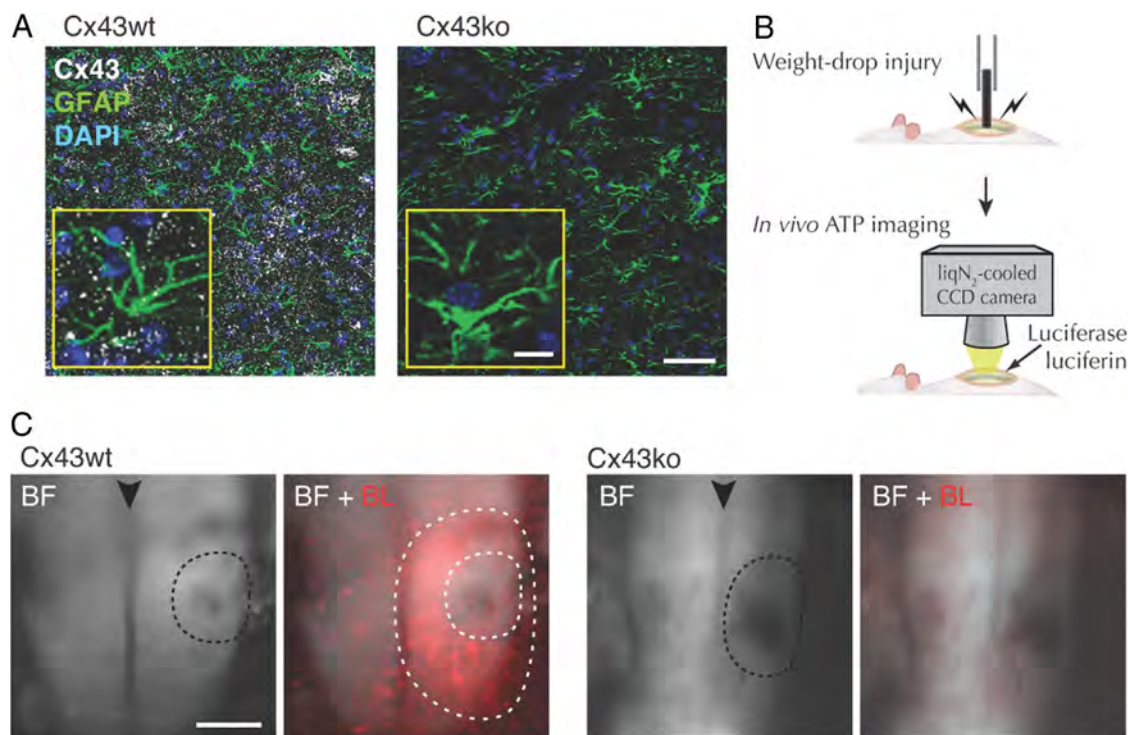
Figure 1. Peritraumatic ATP release after SCI is less in Cx43ko. *A*, Immunolabeling against Cx43 in spinal cord sections from Cx30$^{-/-}$:Cx43$^{fl/fl}$ (Cx43wt) and Cx30$^{-/-}$:Cx43$^{fl/fl}$:hGFAP–Cre (Cx43ko) mice. Scale bar, 50 μm. Inserts, High-magnification images. Scale bars, 10 μm. *B*, A schematic of experimental procedure. *C*, Bioluminescence detection of ATP release from the exposed spinal cord of Cx43wt and Cx43ko mice after SCI. BF, bright-field views of dorsal side with the lesioned regions indicated by dotted lines; black arrowheads indicate dorsal spinal vein. BF + BL, Bioluminescence images (red) superimposed on bright-field images. White dotted lines indicate the area with high ATP release surrounding the lesion. Scale bar, 0.5 mm.

were anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). A laminectomy of the dorsal portion of T11 was performed, and the vertebral column was held with fine clamps at the T10 and T12 levels. The exposed dorsal surface of the spinal cord was subjected to drop of a 3 g weight with flat tip (diameter 0.5 mm) from a height of 12.5 mm (modified NYU impactor) (Peng et al., 2009). Lesions were lateral or on the midline. The Basso Mouse Scale for Locomotion (BMS) rating scale, from 0 (no ankle movement) to 9 (normal gait), was used for evaluating hindlimb movement (Basso et al., 2006; Peng et al., 2009). The mice were blindly evaluated daily at the same time for 8 weeks after injury. ATP release from the exposed spinal cord was imaged by bioluminescence (Wang et al., 2004) within 1 h after SCI. High ATP area was defined as the area with luminescence >1 standard deviation above the average luminescence of the field >2 mm away from the injury.

In vivo *recordings of compound action potentials.* Mice were anesthetized with isoflurane (1.5–2.5%), immobilized with pancuronium bromide (0.3 mg/kg), and artificially ventilated (SAR-830, CWE). A bipolar stimulating electrode (TST33A05KT, WPI) was inserted 150 μm into the left dorsal column rostral to the lesion. Stimulation was delivered through an isolated constant current source (IsoFlex). A saline-filled glass recording electrode was inserted 150 μm into the left dorsal column, 5 mm caudal from the stimulating electrode. One week after SCI, compound action potentials (CAPs) were recorded (20 μs sampling time) by using a 700B amplifier (Molecular Devices) with pCLAMP software, and their amplitude was measured as the difference between positive and negative peaks. White matter loss was evaluated by Luxol Fast Blue (0.1%) staining of cryosections (20 μm). The dorsal column was first identified in sections located at least 1 mm from the lesion border, and then the column was followed on serial sections through the lesion center. The section with the most severe loss of the dorsal column was identified, and the percentage of spared axons was quantified by using ImageJ (National Institutes of Health).

Immunohistochemistry. At 1 week and 1 month after SCI, mice were perfusion fixed and vibratome sections were labeled against GFAP (1:500, monoclonal against purified pig spinal cord GFAP, G3893, Sigma),

ionized calcium binding adaptor molecule 1 (Iba1) (1:500, polyclonal against C terminus, 019–19741, Wako), or CD68 (1:100, monoclonal against ED1 clone, MCA341GA, Serotec). Six fields (200 × 200 μm^2) near the lesion, one each from rostral, middle, and caudal to the lesion, left and right in gray matter, were chosen for analysis. Confocal images were taken at 1 μm steps for 10 μm depth and stacked together before quantification; capture parameters were set from a wild-type spinal cord and remained constant. The average intensity of GFAP and Iba1 were compared with average intensity at least 5 mm away from the lesion in the same section, and the number of CD68+ cells in a field was counted (Peng et al., 2009). Lesion volume was quantified on serial cryosections labeled against GFAP.

Statistics. All data are expressed as mean ± SEM. For statistical evaluations, Kruskal–Wallis test and/or Mann–Whitney test were used when normality was rejected by Shapiro-Wilk test. Otherwise, Student's *t* test was used.

Results

To assess the effect of connexins on ATP release evoked by spinal cord injury, we used mice with *loxP* conditional deletion of Cx43 in astrocytes with Cre expression driven by the hGFAP promoter (Theis et al., 2003). Because deletion of Cx43 can induce increased expression of Cx30 (Wallraff et al., 2006; Lin et al., 2008), adult female littermates with deletion of Cx30 and intact Cx43 expression (Cx30$^{-/-}$:Cx43$^{fl/fl}$ without hGFAP-Cre, here termed Cx43wt) were compared with mice with deletion of Cx30 and conditional deletion of Cx43 (Cx30$^{-/-}$:Cx43$^{fl/fl}$:hGFAP–Cre, here termed Cx43ko). Immunohistochemistry of spinal cord sections identified Cx43 plaques between astrocytes of Cx43wt mice, which were absent in Cx43ko mice (Fig. 1*A*). Bioluminescence imaging of the exposed spinal cord in rats previously showed that ATP is released from large peritraumatic regions bordering a weight drop injury (Wang et al., 2004). To analyze weight-drop
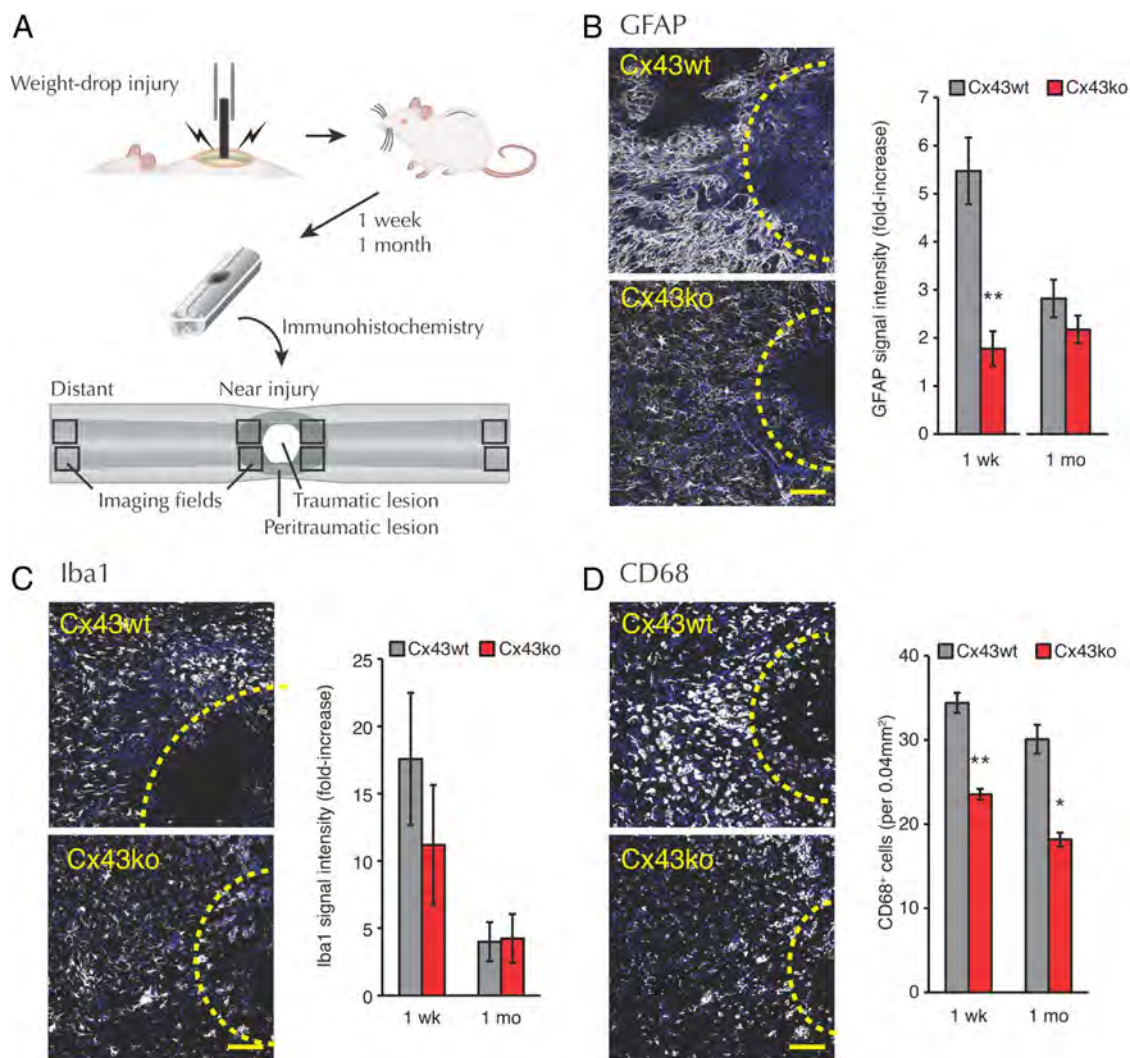
Figure 2. Inflammatory response surrounding the lesion after SCI is less in Cx43ko mice. **A**, A schematic of experimental procedure. **B–D**, Representative images of spinal cord and quantitation from Cx43wt and Cx43ko 1 week after injury: GFAP (**B**), Iba1 (**C**), and CD68 (**D**). Blue, DAPI; white, GFAP, Iba1, or CD68. Yellow dotted lines indicate the position of the lesion. GFAP and CD68 were more upregulated after SCI in Cx43wt than in Cx43ko. Scale bar, 100 μm. $n = 4 - 6$. **$p < 0.01$; *$p < 0.05$, Cx43wt versus Cx43ko.

injury and ATP release in mice, we used a 3 g rod with a 0.5 mm flat tip dropped from a height of 12.5 mm; extracellular ATP was visualized by light emission resulting from ATP-triggered luciferase activity detected by a liquid nitrogen-cooled CCD camera (Fig. 1B). Cx43wt mice exhibited, similar to rats, a sustained increase in ATP release from large regions surrounding the traumatic lesion during the observation period of 10–70 min after the traumatic event (Fig. 1C). In contrast, SCI did not trigger distinct zones of high ATP release in Cx43ko mice (Fig. 1C). The average size of the area with high ATP release was 1.37 ± 0.20 mm^2 ($n = 8$) in Cx43wt and 0.36 ± 0.09 mm^2 in Cx43ko ($n = 5$; $p = 0.003$). This observation indicates that Cx43 plays an important role in ATP release evoked by weight-drop injury of spinal cord.

ATP has previously been implicated as one of several instigators of the innate inflammatory response to traumatic spinal cord injury (Abbracchio et al., 2009). Because there was less ATP release after SCI in Cx43ko mice, we examined whether deletion of Cx43 also reduced post-traumatic inflammation. Spinal cords 1 week or 1 month after SCI were assessed for reactive astrogliosis based on immunolabeling of GFAP (Fig. 2A). At 1 week, there was a 5.5 ± 0.7-fold increase of GFAP immunofluorescent signal in Cx43wt mice close to the traumatic lesion ($n = 6$) compared

with tissue distant from the injury. In contrast, Cx43ko mice exhibited only a 1.8 ± 0.4-fold ($n = 4$) elevation of GFAP around the lesion ($p = 0.004$; Cx43wt vs Cx43ko) (Fig. 2B). At 1 month after injury, GFAP immunoreactivity close to the lesion fell to 2.8 ± 0.4 ($n = 4$) times the level in distant tissue in Cx43wt, whereas this ratio in Cx43ko remained unchanged (2.2 ± 0.3-fold; $n = 4$) ($p = 0.248$; Cx43wt vs Cx43ko). Iba1 has been implicated in the activation and motility of microglia/macrophages. The Iba1 immunofluorescence around the lesion was elevated at both 1 week and 1 month without significant difference between Cx43wt and Cx43ko ($p > 0.5$) (Fig. 2C). In contrast, when activated microglia were counted by labeling for CD68, a lysosomal protein expressed by cells of the monocyte-macrophage lineage, more CD68-positive cells were observed in Cx43wt than in Cx43ko at both 1 week and 1 month after injury (Fig. 2D). Thus, deletion of Cx43 in astrocytes reduced acute astrogliosis and microgliosis, which is consistent with the activation of astrocytes and microglial cells at least in part by ATP release from astrocytes.

Because deletion of Cx43 significantly prevented injury-induced increase in GFAP and CD68, traditional indicators of inflammation, we assessed functional recovery. One week after
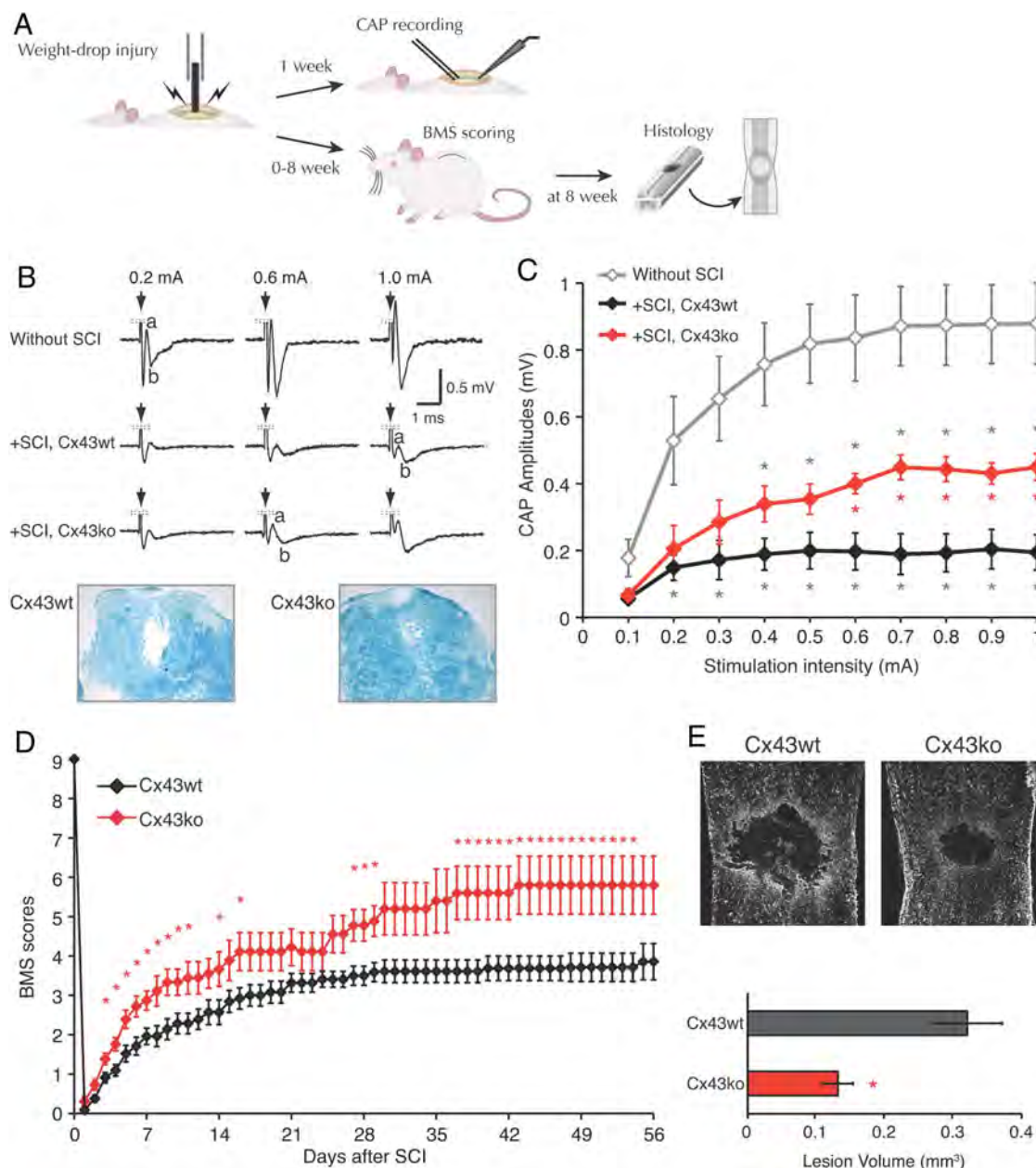
Figure 3. Functional recovery after SCI is facilitated in Cx43ko mice. ***A***, A schematic of experimental procedure. ***B***, Top, Representative tracings of CPAs in spinal cord caudal to the lesion in response to stimulation on the rostral side in Cx43wt with injury (+SCI), Cx43ko with injury (+SCI) 1 week after lesioning, and sham control without SCI. No difference was observed between Cx43wt and Cx43ko without SCI ($p > 0.2$). A large spike by the stimulation artifact was removed from the traces. Bottom, Luxol Fast Blue-stained dorsal column of spinal cord at the lesion center 1 week after the injury in Cx43wt and Cx43ko. ***C***, A summary histogram of CAP amplitudes against stimulation intensity in Cx43wt with injury, Cx43ko with injury, and sham control without injury ($n = 6$). *$p < 0.017$, compared with sham (gray) or between Cx43wt and Cx43ko (red). ***D***, Functional recovery evaluated by BMS locomotor rating, showing improved recovery in Cx43ko ($n = 5$–26) compared with Cx43wt ($n = 7$–29). Score at day 0 was measured just before the injury. *$p < 0.05$. ***E***, Top, GFAP immunostaining images of spinal cords 8 weeks after injury. Bottom, A summary histogram of traumatic lesion size in Cx43wt ($n = 8$) and Cx43ko ($n = 6$) spinal cord. *$p < 0.05$.

injury CAPs that propagated rostro-caudally across the lesion (Fig. 3*A*) were much smaller than in uninjured Cx43wt control, but larger in Cx43ko than in Cx43wt (Fig. 3*B*). With 1-mA stimulation of Cx43wt cords, CAP amplitudes were 0.878 ± 0.121 mV without SCI and 0.195 ± 0.053 mV at 1 week after SCI ($n = 6$; $p < 0.01$). Cx43ko also showed a reduction of CAP amplitude after spinal cord injury, but amplitudes were larger than in Cx43wt. At 1-mA stimulation, the CAP was ~2.3-fold greater in Cx43ko SCI than in Cx43wt SCI (0.450 ± 0.040 mV; $n = 6$; $p < 0.05$) (Fig. 3*C*). Luxol Fast Blue staining showed that $12.1 \pm 1.8\%$ of dorsal white matter myelin was preserved at the site of lesion in Cx43wt,

explaining why the CAP was not completely abolished by SCI (Qiao et al., 2006), whereas Cx43ko showed significantly more staining at the dorsal column ($42.6 \pm 1.8\%$; $n = 3$–5; $p < 0.001$) (Fig. 3*B*). Thus, by this measure Cx43ko exhibited a greater preservation of spinal cord conduction than Cx43wt after a similar traumatic injury. Deletion of Cx43 also promoted faster recovery of locomotor function after SCI. In blinded analysis using the BMS for locomotion, Cx43ko achieved (for the most part significantly) higher BMS scores starting from 3 d after the injury throughout the evaluation period of 8 weeks, reaching a score of 5.8 ± 0.7 corresponding to consistent hindlimb plantar stepping

with some coordination ($n = 5$) (Fig. 3D). Differences that appeared in a rather early phase after the injury may suggest that the structural injury is also less severe in Cx43ko mice. The recovery was near maximal after 6 weeks. In contrast, Cx43wt recovered to 3.5 ± 0.2 at 4 weeks ($n = 11$) and never reached a score of 4.0 during the recovery period (Fig. 3D). At 8 weeks most of the Cx43wt exhibited plantar placing of the paw, but no plantar stepping.

The volume of the traumatic lesion was evaluated in the same animals. In Cx43wt the volume was 0.32 ± 0.05 mm^3 ($n = 8$), whereas in Cx43ko the volume was only 0.13 ± 0.02 mm^3 ($n = 6$; $p = 0.01$) (Fig. 3E). Thus, the recordings of CAPs at 1 week, assessment of locomotor recovery over 8 weeks, and lesion size at 8 weeks all suggest that deletion of Cx43 reduced the severity of traumatic injury and improved recovery.

Discussion

Previous rat studies showed that SCI causes excessive and sustained ATP release from peritraumatic regions and activation of P2RX7 contributes to reactive changes in both astrocytes and microglial cells as well as neuronal injury (Wang et al., 2004; Cotrina and Nedergaard, 2009; Peng et al., 2009). In this study, we show that expression of Cx43 in astrocytes plays a key role in post-traumatic release of ATP. Bioluminescence imaging of the exposed spinal cord demonstrated that weight-drop injury caused a sharp increase in ATP release from large peritraumatic regions in Cx43wt mice, but in Cx43ko mice the area of post-traumatic ATP release was significantly smaller (Fig. 1). Moreover, deletion of Cx43 decreased the inflammatory response to SCI and suppressed astrogliosis and microgliosis as well as tissue loss (Figs. 2B-D, 3E,F). Furthermore, Cx43ko mice recovered motor functions significantly faster and to a greater extent than Cx43wt littermates after SCI (Fig. 3D).

Cx43 expression is upregulated in regions neighboring traumatic lesions in spinal cord (Theriault et al., 1997; Cronin et al., 2008). The neuroprotective effect of deleting Cx43 likely involves multiple processes that involve hemichannels, gap junctions, or both. Lack of Cx43 hemichannels is expected to reduce leakage of cytosolic small molecules, including ATP from astrocytes located in peritraumatic regions, and lack of cell–cell channels would reduce passage of these molecules from neighboring cells, thereby improving astrocytic survival (Cotrina et al., 1998; Parpura et al., 2004). In turn, viable astrocytes would better support neuronal survival and counteract delayed neuronal loss (Faulkner et al., 2004). The suppression of post-traumatic ATP release would reduce ATP-mediated excitotoxic death of neurons and oligodendrocytes by activation of P2RX7s (Wang et al., 2004) and aggravation of secondary injury. P2RX7s are also expressed by microglial cells and infiltrating leukocytes (Collo et al., 1997) and are linked to release of proinflammatory cytokines, including IL-1β (Ferrari et al., 2006). Cytokines are essential parts of the innate inflammatory response and aggravate excitotoxic actions on neurons and oligodendrocytes (Acarin et al., 2000). Finally, gap junctions have been shown to contribute to secondary injury by passage of proapoptotic compounds from dying to otherwise viable gap junction-coupled cells (Lin et al., 1998). Because deletion of Cx43 effectively uncouples astrocytes, it is possible that "bystander death" is reduced and functional recovery thereby improved in Cx43 knockout mice and possibly more so in our Cx30$^{-/-}$:Cx43$^{fl/fl}$:hGFAP-Cre mice. Moreover, deletion of Cx43 affects the expression of multiple other genes (Naus et al., 2000; Iacobas et al., 2004). Thus, it is plausible that, although Cx43

hemichannels provide a direct conduit for ATP release, Cx43 deletion indirectly reduces ATP release.

Our study contributes to the current revision of mechanisms involved in the innate response to tissue injury. Traditionally it was thought that microglial cells were the first line of defense and microglial cells initiated post-traumatic inflammation by release of cytokines and other proinflammatory agents. However, *in vivo* imaging has shown that purinergic receptor activation is both necessary and sufficient for movement of microglial cell processes in response to local laser injury or ATP injection (Davalos et al., 2005; Nimmerjahn et al., 2005). Microglial cells express several purinergic receptors (Koizumi et al., 2007). Deletion or pharmacological blockage of P2RY12 reduces or eliminates movement of microglial cell processes (Haynes et al., 2006; Tozaki-Saitoh et al., 2008), whereas P2RY6 activation is a key determinant of phagocytosis (Koizumi et al., 2007). Moreover, P2RX7 activation triggers maturation and secretion of IL-1β from microglial cells (Di Virgilio et al., 1999). The observation that deletion of Cx43 reduces post-traumatic ATP release, as well as microglial cell activation, supports the idea that astrocytes are first to sense injury and activation of microglial cells is triggered by astrocytic ATP release. In cultures spinal astrocytes respond to FGF-1 by the release of ATP, which activates purinergic receptors, leading to the opening of pannexin hemichannels and ultimately Cx43 hemichannels (Garré et al., 2010).

An important aspect of the study was that functional recovery occurred significantly faster in Cx43ko mice consistent with the smaller traumatic lesions noted in these animals (Fig. 3). The fact that Cx43ko mice displayed significantly better motor functions as early as 3 d after injury suggests that deletion of Cx43 directly protected dorsal tracts, rather than promoted neuronal regrowth. One possible explanation is that the lack of Cx43 reduced the acute inflammatory response, including tissue swelling and secondary ischemic loss of white-matter tracts. Another possibility is that P2RX7 activation in oligodendrocytes directly contributes to loss of myelin and reduction in action potential amplitude due to exposure to ATP (Matute et al., 2007). Significant recovery of function might then be associated with remyelination (Qiao et al., 2006).

Cx43 may represent a novel target for reducing the severity of traumatic spinal cord injury. Although the neuroprotective effect of Cx43 deletion may involve multiple pathways, our analysis suggests that a reduction in excessive ATP release from peritraumatic areas reduces the post-traumatic inflammatory response that negatively affects recovery.

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Neuronal adenosine release, and not astrocytic ATP release, mediates feedback inhibition of excitatory activity

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Adenosine is a potent anticonvulsant acting on excitatory synapses through A1 receptors. Cellular release of ATP, and its subsequent extracellular enzymatic degradation to adenosine, could provide a powerful mechanism for astrocytes to control the activity of neural networks during high-intensity activity. Despite adenosine's importance, the cellular source of adenosine remains unclear. We report here that multiple enzymes degrade extracellular ATP in brain tissue, whereas only Nt5e degrades AMP to adenosine. However, endogenous A1 receptor activation during cortical seizures in vivo or heterosynaptic depression in situ is independent of Nt5e activity, and activation of astrocytic ATP release via Ca^{2+} photolysis does not trigger synaptic depression. In contrast, selective activation of postsynaptic CA1 neurons leads to release of adenosine and synaptic depression. This study shows that adenosine-mediated synaptic depression is not a consequence of astrocytic ATP release, but is instead an autonomic feedback mechanism that suppresses excitatory transmission during prolonged activity.

purinergic signaling | purine | glia | calcium signaling

Several lines of work over the past three decades have documented that adenosine acts as an endogenous anticonvulsant (1–3). The extracellular concentration of adenosine increases during seizures, and it has been proposed that status epilepticus is a result of loss of adenosine signaling (4). Conversely, mice lacking A1 receptors exhibit a decreased threshold for seizure propagation (5–7). Adenosine can be generated in the cytosol of neurons as a consequence of the metabolic exhaustion and released directly, as adenosine, via the equilibrative nucleoside transporters (ENTs)—e.g., the ubiquitously expressed ENT1 and ENT2 (8, 9). Alternatively, adenosine is released indirectly, as ATP followed by extracellular enzymatic catabolism to adenosine. The CNS expresses several ectoenzymes, including nucleoside triphosphate diphosphohydrolases (NTPDases; e.g., CD39, NTPDase-2), ectonucleotide pyrophosphatase/phosphodiesterases (e.g., autotaxin), and ecto-5′-nucleotidases (CD73/Nt5e, prostatic acid phosphatase, and alkaline phosphatase) (10–12). Although the regional and cellular activity patterns for each of these enzymes have not been fully explored, exogenous addition of ATP to ex vivo preparations has shown that all regions of the mammalian brain can dephosphorylate ATP to AMP through an ADP intermediate, whereas dephosphorylation of AMP to adenosine occurs primarily in the striatum and olfactory bulb (11). However, the presence of ectoenzymes does not prove that extracellular conversion of ATP to adenosine plays a physiological role, because extracellular adenosine is rapidly recirculated back into the cytosol by ENT1 and ENT2 (13). Further, ATP is released in small quantities during cell–cell signaling, and the effective diffusion of adenosine is limited because of the potent reuptake mechanisms (14). Thus, it is possible that extracellularly generated adenosine is transported back into the cytosol before reaching pre- or postsynaptic localized A1 receptors. The classical action of extracellular adenosine is to mediate feedback inhibition of presynaptic glutamate release through neuronal adenosine release (9, 15). The alternative possibility is that adenosine is released directly by postsynaptic neurons, in parallel with, but independent of, astrocytic Ca^{2+} signaling. To this end, adenosine elicits postsynaptic depression in the absence of ectoenzymatic activities and ATP release. Similar postsynaptic actions have been described for adenosine in both adrenergic and noradrenergic neuronal systems (16). Given the complexity of purinergic signaling and the potential of ATP to activate multiple downstream purinergic pathways (e.g., ATP and the breakdown products ADP may activate distinct P2 receptors), it is conceivable that elimination of extracellular adenine nucleotides is tightly controlled to avoid cross-talk between P2 and P1 purinergic signaling pathways.

Results

Multiple Enzymes Degrade Extracellular ATP in Brain Tissue, Whereas only Nt5e Degrades AMP to Adenosine. Adenosine acting on its receptors can derive either from cellular release of adenosine or from extracellular degradation of released ATP. In the extracellular space, ATP degradation is catalyzed by multiple ectoenzymes that sequentially hydrolyze ATP to adenosine, whereas AMP-to-adenosine formation is primarily catalyzed by the ectoenzyme Nt5e. To distinguish whether adenosine receptor activation is a consequence of adenosine release or ATP degradation by ectoenzymatic activity, we established an assay using HPLC-UV analysis to identify and quantify the degradation products of exogenously added ATP to brain slices (Fig. 1A). We first confirmed that ATP added to slices accumulated into ADP, AMP, adenosine, and inosine linearly over time, whereas control slices with no ATP added did not accumulate significant amounts of these adenine nucleotides (Fig. S1A). Several enzymes could catalyze the degradation of ATP to ADP and AMP, including the NTPdase CD39. To this end, we first incubated brain slices from CD39 knockout mice (CD39$^{-/-}$) in ATP and found that, whereas ADP accumulated ($144 \pm 28\%$ ADP, $P < 0.0001$), AMP levels were significantly reduced ($31.6 \pm 0.29\%$ AMP, $P < 0.0001$) (Fig. 1B). A similar observation was made by using 6-N, N-diethyl-D-β,γ-dibromomethylene ATP (ARL 67156; 100 μM), a nonselective inhibitor of several NTPdases ($257.6 \pm 9.2\%$ ADP, $39.8 \pm 0.2\%$ AMP, $P < 0.0001$) (Fig. 1B). This result is consistent with previous observations reporting that ARL 67156
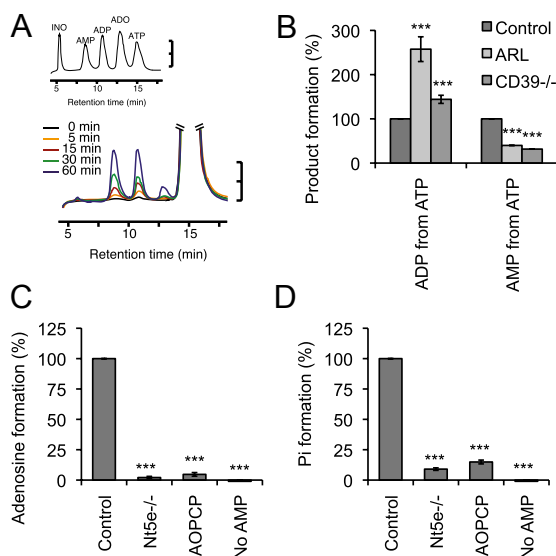
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Fig. 1. Multiple enzymes degrade extracellular ATP in brain tissue, but only Nt5e degrades AMP to adenosine. (*A Upper*) HPLC-UV analysis at 260 nm identified inosine (INO), AMP, ADP, adenosine (ADO), and ATP (each 10 µM). (*Lower*) HPLC-UV analysis of media samples collected over time (0, 5, 15, 30, and 60 min) from brain slice incubated with ATP. ATP accumulated into ADP, AMP, adenosine, and inosine linearly ($r^2 > 0.99$) over time at rates of 2.80 ± 0.76, 2.60 ± 0.13, 0.60 ± 0.26, and 0.24 ± 0.12 nmol/min per slice, respectively (representative experiment). Notably, spontaneous degradation of ATP, ADP, and AMP in media was insignificant in the absence of slices (ATP, 0.057 ± 0.006%; ADP, 0.004 ± 0.003%; AMP, 0.000 ± 0.000% free phosphate accumulation per minute, mean ± SEM). (*B*) Catabolism of ATP in slices in the presence of ARL 67156 or in slices from CD39⁻/⁻ mice, both in the presence of AOPCP. Bars denote percentage of accumulated ADP or AMP from ATP compared with control (mean ± SEM, n = 6–9 slices). (*C*) Formation of adenosine from AMP from wild-type control, Nt5e⁻/⁻ mice, wild type with AOPCP, and wild-type control slices without AMP. Bars denote percentage of accumulated adenosine compared with control (mean ± SEM, n = 9 slices). (*D*) Formation of free phosphate from AMP in similar experimental setup as in *C* (mean ± SEM, n = 9 slices). *P < 0.05; ***P < 0.001; Student *t* test, all compared with wild type.

only partially inhibits ATP dephosphorylation (17–19), suggesting that several NTPdases—including CD39, NTPDase-2 and -3, and alkaline phosphatase—are functional in brain tissue.

We next investigated whether adenosine formed from AMP was exclusively dependent upon Nt5e activity using slices from Nt5e knockout mice (Nt5e⁻/⁻) or wild type incubated in α,β-methylene adenosine 5′-diphosphate (AOPCP; 100 µM), an inhibitor of Nt5e. Wild-type slices incubated in AMP degraded it into adenosine and inosine linearly over time (Fig. S1*B*). This adenosine formation was not due to cellular release, because free phosphate also accumulated (Fig. 1*D*), and adenosine or phosphate accumulation was not observed in the absence of substrate (Fig. 1 *C* and *D*). However, adenosine formation was almost absent when slices from Nt5e⁻/⁻ mice were incubated in AMP (2.10 ± 0.97% ADO, P < 0.0001) or when wild-type slices were incubated in AMP and AOPCP (4.70 ± 0.00% ADO, P < 0.0001) (Fig. 1*C*). This observation was mirrored by a lack of accumulation of free phosphate (Fig. 1*D*). The accumulation of phosphate was slightly higher than that of adenosine, likely reflecting the reuptake of adenosine by nucleoside transporters. All together, this analysis suggests that, although multiple ectoenzymes degrade ATP to AMP in brain tissue, only one enzyme, Nt5e, degrades AMP to adenosine.

Endogenous A1 Receptor Activation During Cortical Seizures in Vivo Is Not a Consequence of Cellular ATP Release.
Activation of adenosine A1 receptors has been shown to exhibit an anticonvulsant effect

(20–22), and endogenous adenosine levels rise during local seizure activity (23), suggesting that adenosine may act through A1 receptors. Although the anticonvulsant effect of adenosine is well known, the cellular source remains unsolved. To evaluate whether adenosine derives from cellularly released ATP, we used the in vivo seizure model by Dichter & Spencer (20) in which a penicillin crystal is deposited onto brain tissue to trigger a seizure. Local field potentials (LFPs) were measured before and after penicillin administration in cortical layer 2 by using two electrodes (Fig. 2*A*). It took 11.8 ± 1.7 min for the high-amplitude, high-frequency discharges characteristic of seizure activity to reach electrode 1 and 14.5 ± 1.8 min to reach electrode 2 (Fig. 2 *B* and *C*). We confirmed that A1 receptor activation suppressed the spread of local seizures, because the spatial expansion of cortical hyperexcitability was nearly twice as fast in mice with either a deletion of A1 receptors (A1R⁻/⁻; ref. 5) (electrode 1: 50% of wild type; electrode 2: 49% of wild type) or in wild-type mice receiving the A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 1 mg/kg i.p.) (electrode 1: 49% of wild type; electrode 2: 61% of wild type; Fig. 2 *B* and *C*). We then evaluated whether the cellular source of adenosine derived from ATP by inhibiting the activity of Nt5e. However, neither AOPCP administration (10 mM, 10 µL injected in cisterna magda) (electrode 1: 108% of wild type; electrode 2: 103% of wild type) nor deletion of Nt5e (Nt5e⁻/⁻ mice) (electrode 1: 121% of wild type; electrode 2: 131% of wild type) decreased the latency of the penicillin-induced focal seizure (Fig. 2 *B* and *C*). These observations were not a result of changes in spike morphology, because the frequency and amplitude of LFP spikes did not differ between the groups (ANOVA, P > 0.1), except that the amplitude of LFP spikes were increased in mice with deletion of A1 receptors (Fig. 2 *D* and *E*). Thus, this analysis suggests that in the setting of focal cortical seizures in vivo, A1 receptor activation is not a consequence of cellular ATP release.

Activity-Dependent Heterosynaptic Depression in Slices Does Not Require Nt5e Activity.
Another model frequently used to study adenosine-mediated suppression of excitatory transmission is heterosynaptic depression of CA1 pyramidal cells induced by high-frequency stimulation (HFS) (15, 24, 25), which causes a depression in adjacent nonexcited neurons in an A1 receptor-dependent fashion (24). It has been suggested that astrocytic Ca²⁺ waves release ATP upon HFS and that the subsequent degradation of ATP to adenosine mediates heterosynaptic depression (26–29). We asked whether astrocytic ATP or cellularly released adenosine mediated the heterosynaptic depression. We monitored Ca²⁺ responses simultaneously with recording of evoked excitatory postsynaptic potentials (eEPSPs) through a recording electrode before and after HFS (Fig. 3*A*) and found that the astrocytic Ca²⁺ wave propagated slowly and reached the impaled neuron with a delay of 30–40 s (Fig. 3*B*). However, the eEPSP amplitude was significantly and rapidly reduced 10 s after HFS (Fig. 3 *E* and *F*). Bath application of DPCPX (300 nM) attenuated synaptic depression (97.7 ± 4.2%, P < 0.0001), suggesting that adenosine A1 receptor activation played a crucial role in heterosynaptic depression (Fig. 3*F*). Notably, the P2 receptor antagonists, pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; 50 mM) and suramine (50 mM), significantly inhibited astrocytic Ca²⁺ waves evoked by HFS [control 85 ± 11 DF/F (%); PPADS 34.9 ± 6.5 DF/F (%), P < 0.01; Suramin 40.7 ± 3.7 DF/F (%), P < 0.05] (Fig. 3*C*) as previously reported (30, 31), suggesting that ATP was indeed present. However, neither of these agents impacted heterosynaptic depression, suggesting that the two events were separate (Fig. 3*D*). Interestingly, application of AOPCP (100 µM) or the use of slices from Nt5e⁻/⁻ mice did not affect the depression of eEPSPs after HFS (AOPCP 68.4 ± 4.2% of baseline before HFS; Nt5e⁻/⁻ 59.7 ± 4.9%), suggesting that the endogenous source of adenosine was not a consequence of cellular ATP release (Fig. 3*F*). Perfusion of DPCPX completely abolished the suppressive effect of HFS on neurons, both in the presence of AOPCP (94.4 ± 4.3%, P < 0.0001) or when slices were prepared from Nt5e⁻/⁻ mice (101.7 ± 7.2%, P <
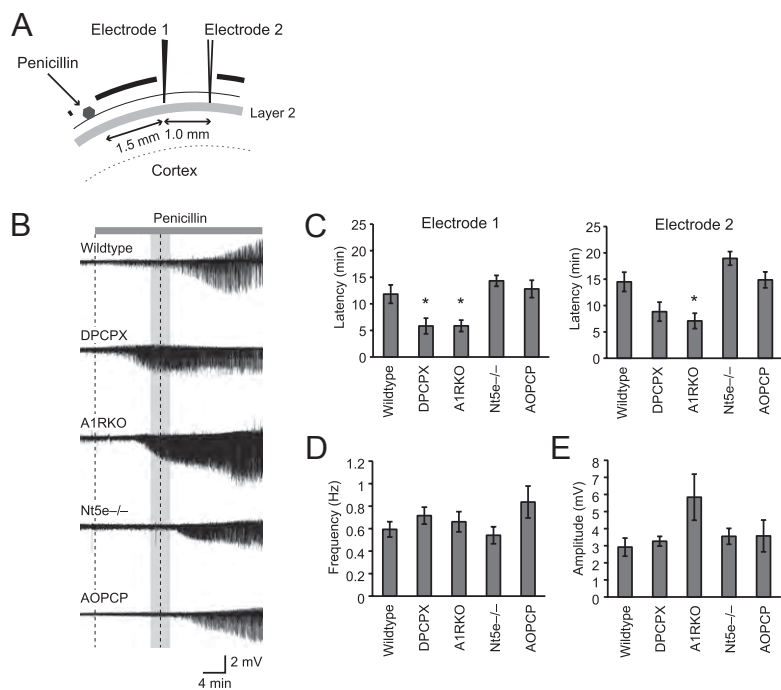
Fig. 2. Endogenous A1 receptor activation during cortical seizures in vivo is not a consequence of cellular ATP release. (*A*) Focal seizures were induced in adult mice by placing a sodium penicillin crystal on the exposed cortical surface. The local spread of hyperexcitability was monitored by two extracellular recording electrodes placed at a distance of 1.5 and 2.5 mm from the site of seizure initiation. (*B*) Representative traces from wild type, wild type with DPCPX, A1R$^{-/-}$, Nt5e$^{-/-}$, and wild type with AOPCP. The position of the dotted line in the middle of the gray box indicates the average latency of 11.8 ± 1.7 min (mean ± SEM) between application of sodium penicillin and arrival of seizure activity at first recording electrode site in wild-type mice (*n* = 9). (*C*) Histogram comparing the latency (minutes) in the five groups (*P < 0.05, ANOVA with Newman–Keuls test, *n* = 9 animals). (*D* and *E*) Histograms comparing maximal frequency and maximal amplitude of neuronal spiking activity detected within the 30-min observation period after application of sodium penicillin (frequency, *P* = 0.24; amplitude, *P* = 0.10, ANOVA, *n* = 9 animals).

0.0001), suggesting that adenosine also mediated heterosynaptic depression in the absence of Nt5e activity (Fig. 3*F*). All together, the independence of Nt5e activity on heterosynaptic depression suggests that synaptic depression is mediated by direct release of adenosine and not by ATP.

Selectively Inducing Astrocytic ATP Release via Ca^{2+} Photolysis Does Not Trigger Synaptic Depression. Because several reports suggest that astrocytic Ca^{2+}-mediated ATP release is the source of A1 receptor-mediated synaptic depression, we next selectively activated Ca^{2+} signaling in astrocytes using photolysis of nitrophenyl (NP)-EGTA caged Ca^{2+} (10 μM) (28, 32–34) while monitoring both astrocytic Ca^{2+} signaling and the effect on eEPSPs (Fig. 4*A*). The advantage of photolysis is that astrocytes are selectively stimulated without interfering with synaptic transmission. NP-EGTA was uncaged in a single target located ∼60 μm from the CA1 neuron used for whole-cell recordings. Uncaging triggered an immediate Ca^{2+} increase in the target astrocyte, which slowly propagated by engaging surrounding astrocytes to a maximal radius of ∼100 μm (Fig. 4*B*). We found that the amplitude of evoked EPSP amplitude was not depressed immediately after uncaging (99.9 ± 5.7% at ∼10 s after uncaging compared with before uncaging) because Ca^{2+} increases were evident in astrocytes surrounding the impaled neuron (104 ± 12% at ∼20 s after uncaging and 111.7 ± 7.3% at ∼30 s after uncaging) or as the Ca^{2+} wave passed the impaled neuron (118.5 ± 5.3% at ∼40 s after uncaging) (Fig. 4*C*). This finding suggests that astrocytic Ca^{2+} signaling is not associated with A1 receptor-mediated synaptic depression.

Selectively Activating Postsynaptic CA1 Neurons Triggers Release of Adenosine and Synaptic Depression. Because our experiments excluded extracellular ATP as a source of adenosine, our next question was to establish which cell type(s) release adenosine. Previous studies have suggested that adenosine is released directly, as adenosine, from neurons providing local feedback inhibition in response to excessive firing and metabolic exhaustion (8, 9, 15). However, these studies used HFS, which in addition to excessive firing also triggers Ca^{2+} signaling in astrocytes (Fig. 3) (30, 31). We used a unique approach to address whether neurons release adenosine through equilibrative transporters in the absence of astrocytic activation. Single neurons were whole-cell patch clamped, and a depolarizing current lasting 1 s was delivered at 10-s

intervals, which on average triggered 10 action potentials over 30 repetitive stimulations (Fig. 5 *A* and *D*). This stimulation protocol did not cause a detectable increase in astrocytic Ca^{2+} levels, consistent with prior reports indicating that astrocytes are not activated by postsynaptic activity (35, 36) (Fig. 5 *B* and *C*). Comparison of the eEPSP amplitudes recorded before and immediately after the train of action potentials revealed that stimulation was linked to a sharp reduction in amplitude (40.7 ± 4.8% reduction after repeat firing, *P* < 0.0001). The suppression of eEPSP amplitude was a consequence of A1 receptor activation, because DPCPX (300 nM) potently attenuated the activity-induced reduction of eEPSP amplitude (1.3 ± 2.7% reduction after repeat firing, *P* = 0.5) (Fig. 5*E*). Deletion of Nt5e (Nt5e$^{-/-}$ mice) did not attenuate the depression of the eEPSP amplitude (45.3 ± 2.6% reduction after repeat firing, *P* < 0.01), thus eliminating extracellular catabolism of ATP as the source of adenosine. Rather, adenosine might be generated in the cytosol of neurons and released directly, as adenosine, through the transporters ENT1 and ENT2 (8, 9). The traditional inhibitors of ENT1 and ENT2, including dipyramidole and nitrobenzylthioinosine, are membrane permeable (37) and may leak out if administered intracellularly and thereby inhibit adenosine transporters in surrounding cells. However, because ENT1 and ENT2 transport both adenosine and inosine with approximately equal affinity (37, 38), we used inosine as a competitive inhibitor of adenosine efflux (38, 39). Strikingly, activity-dependent depression of synaptic transmission was completely blocked when inosine was added to the patch pipette solution to block efflux of cytosolic adenosine (3.9 ± 9.0% reduction after repeat firing, *P* = 0.88) (Fig. 5*E*). To evaluate the maximal potency by which adenosine inhibited eEPSP amplitude, we superfused the slice with 100 μM adenosine and found a depression of the same magnitude as when we used HFS. In contrast, superfusion of 100 μM inosine had no effect. Notably, neither of the manipulations involving DPCPX, Nt5e$^{-/-}$, or inosine affected the number of action potentials generated during the 1-s pulse of depolarization (Fig. 5*F*). Combined, these observations provide direct evidence that excessive spiking is associated with an increase in the cytosolic adenosine concentration in single neurons, which, following facilitated efflux by membrane transporters, inhibits excitatory input via A1 receptors.
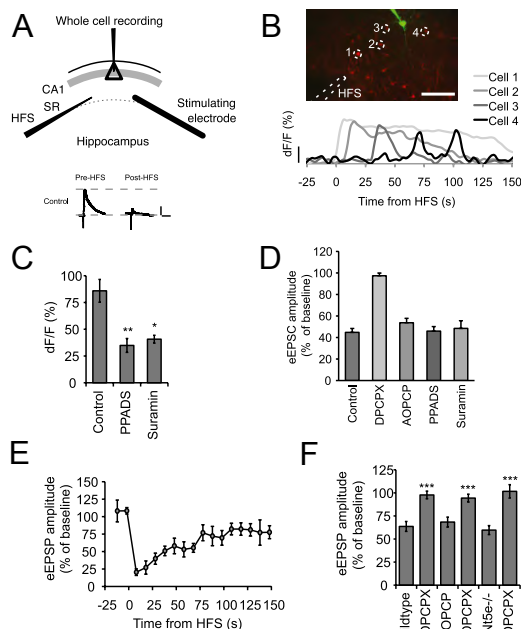
Fig. 3. A1-mediated synaptic depression in the hippocampus is not a consequence of cellular ATP release and precedes astrocytic ATP release. (*A*) Heterosynaptic depression was induced in hippocampal CA1 neurons by using two electrodes: one stimulating electrode to evoke eEPSPs in one afferent pathway (100 μA for 100 us every 10 s) and the HFS electrode to deliver HFS in a separate location that was independent of the first afferent pathway (200 μA 100 Hz for 1 s, 100 pulses). (Scale bars: vertical, 4 mV; horizontal, 4 ms.) (*B*) HFS induced Ca^{2+} wave propagation in Rhod-2 loaded astrocytes (red). The patched neuron is loaded with Alexa 488 (green). [Scale bars: 40 dF/F (%); image, 100 μm.] (*C*) The P2 receptor antagonists, PPADS or suramine, partly inhibited Ca^{2+} wave propagation. (*D*) HFS induced heterosynaptic depression in the presence of DPCPX, AOPCP, PPADS, and suramin. (*E*) HFS induced heterosynaptic depression as evidenced by a depression in the eEPSPs (filled circles; $n = 7$, mean ± SEM). Representative eEPSP traces are shown in *A*. (*F*) Histogram shows effect of AOPCP in wild-type slices ($n = 6$) or slices from Nt5e$^{-/-}$ mice ($n = 6$) with or without DPCPX ($n = 5$) on depressed. Student t test, *$P < 0.05$; **$P < 0.01$.

Discussion

In the present study, we show that active spiking neurons release adenosine through ENTs, leading to suppression of excitatory transmission. We propose that this mechanism functions as a fatigue feedback signal to prevent metabolic exhaustion under high-intensity activity, which otherwise would lead to uncontrolled neuronal signaling. Using acute brain slices, we first showed that only one ectoenzyme, Nt5e, catalyzed extracellular adenosine formation from AMP (Fig. 1). Using this information, we next dissected the role of Nt5e in physiological activation of the A1 receptor in vivo and in acute brain slices. Our analysis showed that neither genetic deletion nor pharmacological inhibition of Nt5e played a role in A1 receptor activation, suggesting that adenosine is not generated in the extracellular space from ATP, but rather is released directly (Figs. 2 and 3). Moreover, uncaging of caged Ca^{2+} triggered slowly propagating astrocytic Ca^{2+} wave, but did not inhibit excitatory transmission (Fig. 4). However, when we selectively increased firing of a single excitatory neuron, A1 receptors were activated in an ENT-dependent and Nt5e-independent fashion (Fig. 5), suggesting that active spiking neurons release adenosine. Together, these data demonstrate that, although astrocytic ATP is released simultaneously with seizure activity in vivo and HFS in acute brain slices, ATP is not degraded into adenosine in sufficient quantities to cause A1 receptor-mediated synaptic depression.
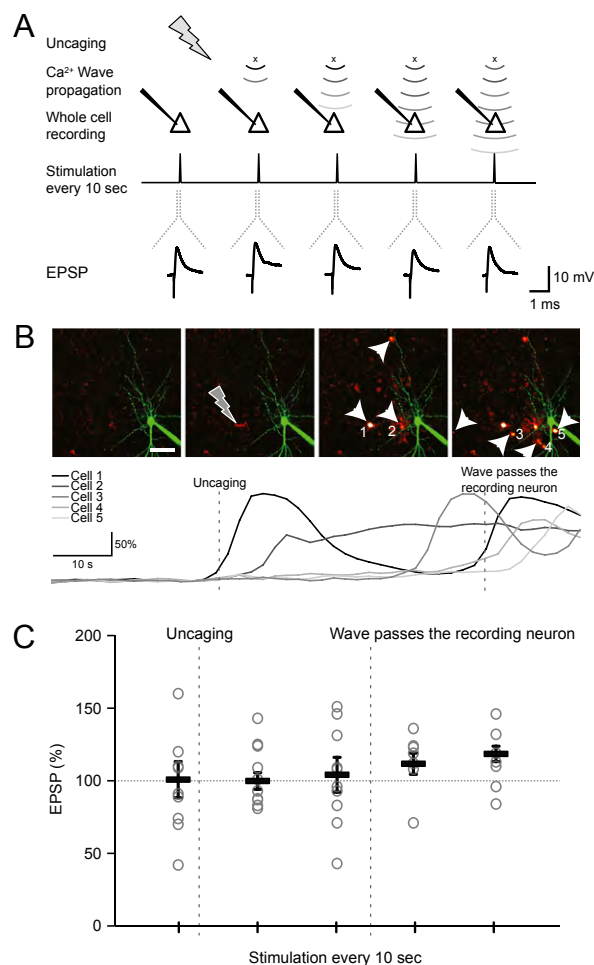

Fig. 4. Selectively activating astrocytic calcium does not affect excitatory transmission. (*A*) Whole-cell recordings in CA1 pyramidal neurons with eEPSPs evoked every 10 s. (*B Upper* panel) Astrocytes loaded with the Ca^{2+} indicator Rhod-2/am (3 μM) (red) and caged Ca^{2+} (NP-EGTA/am, 10 μM) was targeted by UV flash, which elicited a propagating Ca^{2+} wave that passed the impaled neuron (green). (Scale: 50 μm.) (*Lower* panel) Traces represent Ca^{2+} changes over time in individual cells as shown in *Upper* panel. (*C*) The eEPSP amplitude before photolysis and during the propagation of the Ca^{2+} wave was used for detecting local increases in extracellular adenosine concentration. The astrocytic Ca^{2+} waves failed to suppress the amplitude of the eEPSP (mean ± SEM, $n = 7$, Student t test).

We suggest that in prior studies, using exogenous addition of ATP has exhausted adenosine reuptake and artificially flooded the extracellular space with adenosine. In other words, exogenous addition of ATP created extracellular signaling pathways that are not active when ATP is released in much smaller quantities during physiological signaling among astrocytes or other cell types. Our data do not exclude that astrocytes by other mechanisms regulate the extracellular concentration of adenosine and thereby contributes to seizure pathogenesis. Reuptake of extracellular adenosine via ENTs (ENT1 and ENT2) is controlled by adenosine kinase (ADK), an enzyme that predominantly or exclusively is expressed in astrocytes adult brain (40). ADK expression is increased in reactive astrocytes and glia scar tissue (41).

Several studies have concluded that ATP released by a Ca^{2+}-dependent vesicular mechanism is responsible for adenosine-mediated synaptic depression. However, the link between extracellular ATP and adenosine was not directly analyzed, because these studies manipulated vesicular release, astrocytic metabolism, or partially inhibited NTPDase activity (24, 25). Despite the fact that these manipulations occurred several steps upstream of the
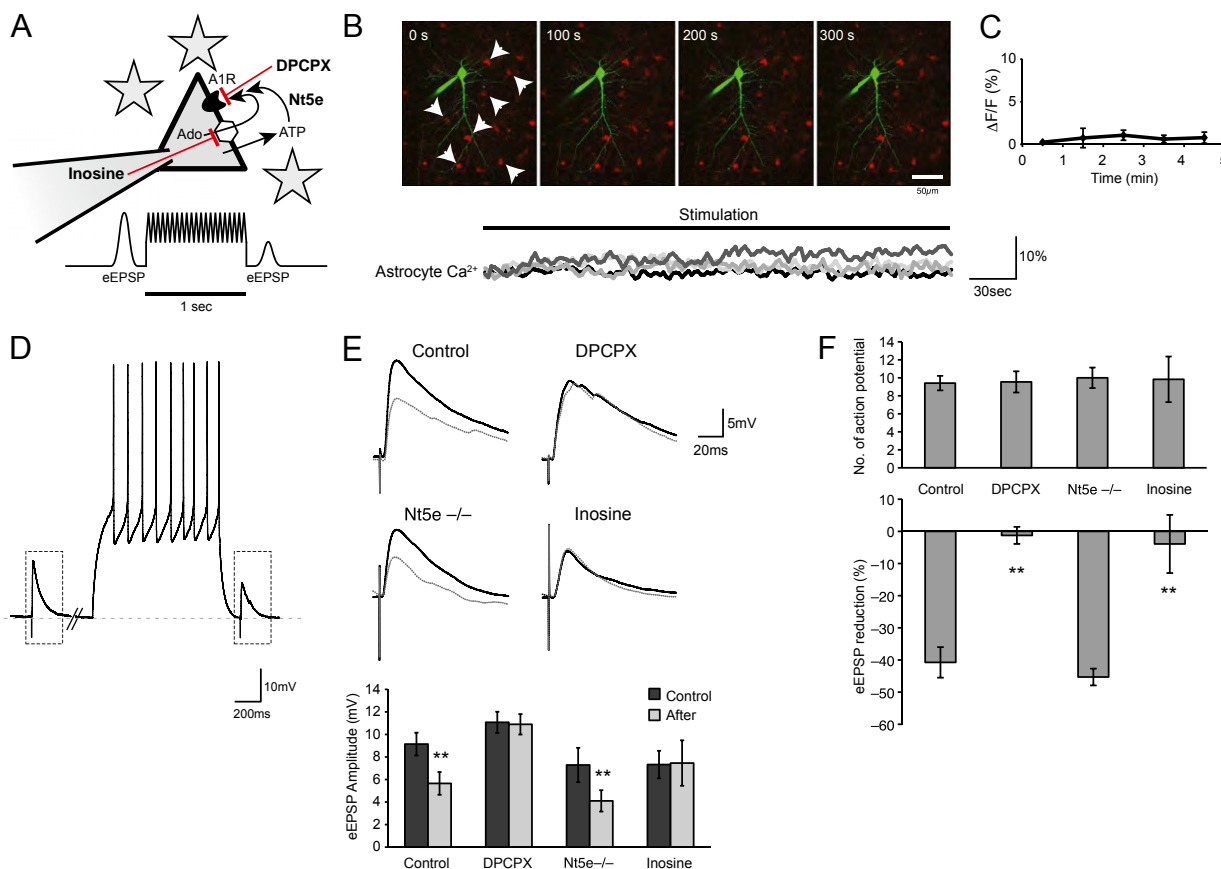
Fig. 5. Selectively activating excitatory neurons triggers release of adenosine and synaptic depression. (*A*) Selective stimulation of CA1 hippocampal neurons and targeting of adenosine metabolism. The impaled CA1 neuron was stimulated by injecting the minimal current needed to induce repeated action potentials, and synaptic depression was assessed by comparing eEPSP amplitude before and immediately after depolarization. (*B*) A train of 30 depolarization pulses in the impaled neuron (green) failed to trigger Ca^{2+} increases in surrounding Rhod-2/am loaded astrocytes (red). (*C*) Average Ca^{2+} increases in astrocytes ($n = 11$). (*D*) Representative trace of eEPSPs before and after (indicated by dotted boxes) the 1-s depolarization. (*E Upper*) Representative eEPSP traces before (black line) and after (gray line) the train of action potentials in the presence of DPCPX, in slices prepared from Nt5e$^{-/-}$ mice, and when inosine (a competitive substrate for the equilibrative nucleoside transporters) was added to the pipette solution. (*Lower*) Summary of several eEPSP traces ($n = 5$–11, ANOVA before repeat firing, $P = 0.1$). (*F Upper*) Number of action potentials elicited during stimulation ($n = 5$–11). (*Lower*) Percentage change in eEPSP amplitude between before and after stimulation. **$P < 0.01$, ANOVA. (Scale bar: 50 μm.)

putative pathway and mainly affected P2 purinergic pathways, the studies concluded that astrocytic ATP is the source of adenosine. In contrast, other studies have concluded that excitatory or inhibitory neurons release adenosine directly to mediate inhibition of postsynaptic hippocampal neurons (8, 15).

In our study, we used several alternative approaches to establish whether synaptic depression was a consequence of ATP or adenosine release. Using an HPLC assay, we found that, although ATP is degraded into AMP by multiple ectonucleotidases in live slices, only one ectoenzyme, Nt5e, degrades AMP into adenosine. Based on this information, we tested whether extracellular ATP can serve as a source of adenosine, using a specific Nt5e inhibitor, as well as transgenic mice knocked out for *Nt5e*. However, Nt5e did not play a role in adenosine-mediated heterosynaptic depression in acute brain slices, suggesting that depression of excitatory transmission is not a consequence of ATP release followed by adenosine formation in the extracellular space. We also used an in vivo model in which the expansion of local seizure was suppressed by A1 receptor activation. We confirmed that inhibition of A1 receptors, but not inhibition of Nt5e activity, facilitated the spread of seizure activity, adding further support to the conclusion that adenosine is released directly from hyperexcitable neurons to mediate feedback inhibition of presynaptic glutamate release and postsynaptic firing as originally proposed (9, 15) (Fig. 2). Across all preparations and ages studied, we consistently found that Nt5e did not contribute to adenosine-mediated A1 receptor activation consistent with findings that Nt5e does

not undergo either a major up- or down-regulation in the postnatal rodent brain (42). This finding is despite the fact that we used adult mice for the in situ enzymatic slice experiments (8–16 wk) and in vivo penicillin-induced seizure experiments (8–12 wk), whereas we were forced to use 13- to 18-d-old mice in our in situ electrophysiology experiments, because slices from older mice do not load well with Ca^{2+} indicators. To account for a potential age-related difference, an analysis of the effect of the A1 receptor antagonist was included in all experiments to assure that adenosine and A1 receptor activity were present in all mice used. Finally, we selectively activated neurons and disrupted their adenosine release using inosine as a competitive substrate to the ENT1 and ENT2 nucleoside transporters. This finding showed that neurons release adenosine directly as a feedback consequence of excessive excitatory activity.

Several lines of evidence have demonstrated ENT1 and/or ENT2 expression in neurons. First, microarray data from FACS-purified neurons show that both developing (P7 and P16) and adult (older than P20) neurons express ENT1 and ENT2 (43, 44). Second, in situ hybridization studies in rat brain tissue demonstrate that cortical tissue and the hippocampal CA1 region express ENT1 (45, 46). We used this information to manipulate adenosine release from CA1 neurons by administering inosine intracellularly as a competitive substrate to adenosine. In conclusion, the source of adenosine depressing neuronal activity during excessive firing is from neuronally released adenosine, and not ATP from astrocytes. We propose that this feedback loop of autocrine adenosine release

functions as a fast and tightly self-regulated mechanism to prevent metabolic exhaustion and potential fatal consequences for excitatory neurons during high intensity activity.

Materials and Methods

Enzymatic assay by HPLC-UV analysis. Enzymatic activities were assessed by placing brain slices from 8–16-wk-old mice in HEPES buffer containing ATP or AMP, and collecting media samples over time that was analyzed for purinergic metabolites using HPLC-UV analysis.

Penicillin-induced seizures. Two cranial boreholes were prepared over parietal cortex in 8–12-wk-old mice. LFP signals were recorded using micro electrodes. Maximal frequency and maximal amplitude discharges were calculated as peak activities during the 30-min recording.

Electrophysiology. Hippocampal pyrimidal neurons were patch-clamped in acute brain slices from 13–18-d-old mice submerged in aCSF. eEPSPs were elicited using a stimulating electrode inserted into the stratum radiatum.

Calcium imaging. A custom-built two-photon laser scanning microscope attached to Tsunami/Millinium laser and a scanning box using Fluoview software was used for imaging Ca^{2+} changes in Rhod-2/am loaded astrocytes. Caged Ca^{2+} compound NP-EGTA/am was uncaged using a UV-laser.
See *SI Materials and Methods* for detailed information.

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ASN NEURO · REVIEW ARTICLE

Neurological diseases as primary gliopathies: a reassessment of neurocentrism

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ABSTRACT

Diseases of the human brain are almost universally attributed to malfunction or loss of nerve cells. However, a considerable amount of work has, during the last decade, expanded our view on the role of astrocytes in CNS (central nervous system), and this analysis suggests that astrocytes contribute to both initiation and propagation of many (if not all) neurological diseases. Astrocytes provide metabolic and trophic support to neurons and oligodendrocytes. Here, we shall endeavour a broad overviewing of the progress in the field and forward the idea that loss of homoeostatic astroglial function leads to an acute loss of neurons in the setting of acute insults such as ischaemia, whereas more subtle dysfunction of astrocytes over periods of months to years contributes to epilepsy and to progressive loss of neurons in neurodegenerative diseases. The majority of therapeutic drugs currently in clinical use target neuronal receptors, channels or transporters. Future therapeutic efforts may benefit by a stronger focus on the supportive homoeostatic functions of astrocytes.

Key words: aging, astrocyte, brain, calcium, disease, supportive cell, transmitter.

INTRODUCTION

Astrocytes are specialized glial cells that are ubiquitously present in all regions of the CNS (central nervous system). Although astrocytes were first identified over 120 years ago and their responses to CNS injury have been recognized for over a century (Kettenmann and Verkhratsky, 2008), for much of this time they have been regarded largely as passive cells providing structural support for neuronal networks; and the notions that astrocytes might make important contributions to CNS function, or that dysfunctions of astrocytes might contribute to CNS pathological remodelling and disease, were generally not considered. In spite of this long 'passive' history, ideas about astrocytes are now changing radically. A steadily growing body of data has over the last 25 years established a variety of essential functions for astrocytes in the healthy nervous system and in the response to injury and disease. Moreover, mechanisms are being elucidated through which the loss or gain of astrocyte functions can contribute to dysfunction or degeneration as well as repair and post-disease remodelling of the CNS. Based on this information, the multiple roles of astroglia, which determine the progression and outcome of neurological diseases, are emerging, and it is becoming clear that astrocytes are involved in various aspects of disease initiation, progression and

resolution. Knowledge emerging about many different aspects of neuropathologies clearly indicates that astrocytes contribute to many neurological diseases and in some cases, such as Alexander disease, is the direct cause of the neurodegeneration. As the astrocyte contribution to specific clinico-pathological entities is being defined, it is useful to compare the astroglial involvement across a number of diseases. We will here specifically review the role of astrocytes in epilepsy, stroke and neurodegenerative diseases. Alexander disease presents clinically as a classical early childhood leucodystrophy with seizure, myelin loss and neuronal degeneration, but is caused by mutation and accumulation of GFAP (glial fibrillary acidic protein). The fundamental fact that a defect in astrocytes underlies dysfunction and death of neurons and oligodendrocytes is discussed.

ASTROCYTES CONTROL CNS HOMOEOSTASIS

Astrocytes are the most numerous, morphologically hetero-geneous and functionally diverse neuroglial cells. Indeed, even the definition of astrocyte does not really exist, as the 'astroglia' as a cell class covers all non-myelinating macroglial cells in the CNS, which along with classical protoplasmic and fibrous astrocytes includes, for example, such different cellular entities as retinal Muller radial glial cells, tanycytes in hypothalamus, pituicytes in the neuro-hypophysis, and ependymocytes, choroid plexus cells and retinal pigment epithelial cells that line the ventricles or the subretinal space (Reichenbach and Wolburg, 2005; Verkhratsky and Butt, 2007). All these cells have, however, one thing in common: their main function is in providing for CNS homoeostasis and therefore astrocytes can be broadly defined as 'homoeostatic neuroglial cells'.

Astrocytes participate in controlling CNS homoeostasis at many levels. Astroglia is critical for maintaining molecular homoeostasis (astrocytes regulate concentra-tions of ions, neurotransmitters and neurohormones in the CNS (Newman, 1995; Danbolt, 2001); metabolic homoeo-stasis (astroglia accumulate energy substrates and supplies neurones with lactate; Magistretti, 2006), cellular hom-oeostasis (astrocytes are directly involved in neurogenesis; Alvarez-Buylla et al., 2001), morphological homoeostasis (astroglia define neural cell migration during development, control synaptogenesis/synaptic pruning and shape the micro-architecture of grey matter (Nedergaard et al., 2003; Pfrieger, 2009), and organ homoeostasis (astroglia control the formation and maintenance of the blood–brain barrier; Abbott, 2005). Furthermore, astrocytes appear to be critically important for brain chemosensing, being able to detect systemic fluctuations in CO_2, pH and Na^+ and initiate behavioural homoeostatic programmes (Shimizu et al., 2007; Gourine et al., 2010; Huckstepp et al., 2010; Gourine and Kasparov, 2011).

Astrocytes also act as integrators in the CNS. In the grey matter, astrocytes create relatively independent neuro-vascular units connected to the capillaries via astroglial perivascular processes. Recent findings show that astrocytes produce and release various molecular mediators, such as prostaglandin E, nitric oxide and arachidonic acid, which can increase or decrease CNS blood vessel diameter and blood flow in a co-ordinated manner (Gordon et al., 2007; Iadecola and Nedergaard, 2007). Furthermore, astrocytes appear to be primary mediators of changes in local CNS blood flow in response to changes in neuronal activity (Schummers et al., 2008; Wolf and Kirchhoff, 2008; Koehler et al., 2009). Astrocytes are fundamental for synaptic transmission and synaptic plasticity. Astrocyte processes that envelop or are in close proximity to synapses provide for spatial specificity of synaptic inputs through isolating individual synapses with astroglial 'cradle' and maintain the fluid, ion, pH and transmitter homoeostasis and provide local metabolic support that are critical for synaptic transmission (Bourne and Harris, 2008; Nedergaard and Verkhratsky, 2012). Astrocytes are endowed with multiple neurotransmitter receptors that allow them to monitor neuronal activity (Verkhratsky, 2010; Lalo et al., 2011a, 2011b) and, moreover, astrocytes secret multiple neurotransmitters and neurohormones (e.g. ATP/purines, glutamate, D-serine, etc.: Halassa et al., 2007a, 2007b; Shigetomi et al., 2008; Perea et al., 2009) that can regulate functional activity of many tens of thousands of synapses located within astroglial territorial domain by a paracrine route. As already mentioned above, astrocyte-derived mole-cules appear to play critical roles in the formation, maintenance and pruning of synapses (Christopherson et al., 2005; Stevens et al., 2007; Barres, 2008). In addition, polypeptide cytokines such as tumour necrosis factor-α produced by astrocytes as well as microglia can influence homoeostatic synaptic scaling by inducing the insertion of AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors at post-synaptic membranes (Stellwagen and Malenka, 2006). Astrocytes are also critical for maintaining glutamatergic transmission by recycling glutamate through the glutamate–glutamine shuttle for subsequent reconver-sion into active transmitter in the synaptic terminal (Danbolt, 2001; Sattler and Rothstein, 2006).

GENERAL PATHOPHYSIOLOGY OF ASTROGLIA: NEUROPATHOLOGY AS HOMOEOSTATIC FAILURE

Every disease of the CNS can be regarded as a homoeostatic failure either inherited (loss of homoeostatic function) or acute (trauma or stroke) or chronic (neurodegeneration). The initiation and progression of these diseases are determined by the degree of homoeostatic loss, which can be either general or function-specific. The general failure is characteristic for acute

insults (trauma, stroke or toxic attack), which compromise brain homoeostasis on many levels from organ (disruption of blood–brain barrier) to metabolic (shutting down the brain energetics) and molecular (loss of neurotransmitter homoeostasis with subsequent excitotoxicity). The specific homoeostatic failures are operative in many forms of chronic pathology, when for example down-regulation of glutamate transporters induces neuronal death in Wernicke encephalopathy. In many pathologies, the homoeostatic failure progresses and broadens when for example initial disruptions of protein catabolism trigger inflammatory reactions, initiate cytotoxicity and compromise the blood–brain barrier (as happens in various neurodegenerative processes).

The systemic homoeostatic function of astroglia makes these cells the main targets for neuropathology (Giaume et al., 2007; Verkhratsky and Parpura, 2010). Astrocytes form the brain defence system by virtue of many homoeostatic molecules expressed in astroglia; using these molecules, astrocytes contain the damage and sustain neuronal survival through maintaining CNS homoeostasis. One of many examples is represented by maintaining brain metabolism following ischaemia/hypoglycaemia, when astrocyte glycogen breaks down to lactate that is transferred to adjacent neural elements where it is used aerobically as fuel (Brown and Ransom, 2007; Brown et al., 2007; Suh et al., 2007). When and if astroglial homoeostatic mechanisms are exhausted the nervous tissue dies.

At the same time all glial homoeostatic mechanisms are endowed with an inherent dichotomy – being developed as survivalistic and protective they may turn to be deleterious and toxic. In conditions of severe insult astrocytes can assume damaging and toxic proportions (Figure 1). For example aquaporins expressed in astroglia are critical for water movements through brain tissue, but they can also be instrumental in mediating oedema in pathology (Zador et al., 2009). Similarly, K^+ channels responsible for potassium buffering when under pathological stress can add to accumulation of extracellular K^+ and mediate spreading depression (Nedergaard and Dirnagl, 2005). The connexins, which connect astrocytes into multicellular syncytia can become the passages for death signals underlying the spread of necrosis through ischaemic penumbra (Lin et al., 1998), whereas depolarization and Na^+ accumulation in astroglia triggers reversal of glutamate transporters which increase glutamate excitotoxicity.

ASTROCYTE RESPONSES TO INJURY AND DISEASE: REACTIVE ASTROGLIOSIS AND SCAR FORMATION

Astrocytes respond to all forms of CNS injury and disease through a process known as reactive astrogliosis. Substantial progress has been made recently in determining functions and mechanisms of reactive astrogliosis and in identifying roles of reactive astrocytes in CNS disorders and pathologies (Figure 2). For the sake of clarity, it is useful to begin with a working definition of the terms 'reactive astrogliosis' and 'glial scar formation' (Sofroniew, 2009; Sofroniew and Vinters, 2010). These terms are not synonymous. Contrary to commonly held beliefs, reactive astrogliosis is not a simple all-or-none stereotypic phenomenon. Instead, reactive astrogliosis is a finely gradated continuum of changes that occur in context-dependent manners regulated by specific molecular signalling events. These changes range from reversible alterations in gene expression and cell hypertrophy with preservation of cellular domains and tissue structure, to long lasting scar formation with rearrangement of tissue structure (Sofroniew, 2009; Sofroniew and Vinters, 2010). Based on a large body of observations in experimental animals and human pathological specimens, a definition of reactive astrogliosis has recently been proposed includes several grades of severity of reactive astrogliosis and glial scar formation that may be encountered in experimental and clinical histopathological examinations (Sofroniew, 2009; Sofroniew and Vinters, 2010). Although the increasing severities of reactive astrogliosis transition seamlessly along a continuum, it is convenient for purposes of description and classification to recognize several broad categories.

Mild to moderate reactive astrogliosis consists of changes (up- or down-regulation) in gene expression and hypertrophy of cell body and processes without substantive loss of individual astrocyte domains and little or no astrocyte proliferation; up-regulation of GFAP expression is prominent. This type of response is generally associated with mild non-penetrating and non-contusive trauma, diffuse innate immune activation (viral infections, system bacterial infections) and areas that are some distance to focal CNS lesions. Because there is little or no reorganization of tissue architecture, if the triggering mechanism is able to resolve, then mild or moderate reactive astrogliosis exhibits the potential for resolution in which the astrocytes return to an appearance similar to that in healthy tissue (Sofroniew, 2009; Sofroniew and Vinters, 2010).

Severe diffuse reactive astrogliosis also consists of changes (up- or down-regulation) in gene expression with pronounced up-regulation of GFAP expression and hypertrophy of cell body and processes, but in addition includes astrocyte proliferation and loss of individual astrocyte domains with substantive intermingling and overlapping of neighbouring astrocyte processes. These changes can result in long-lasting reorganization of tissue architecture that can extend diffusely over substantive areas. This type of response is generally found in areas surrounding severe focal lesions, infections or areas responding to chronic neurodegenerative triggers, and because there can be considerable tissue reorganization, the potential for resolution and return to normal structure is reduced (Sofroniew and Vinters, 2010).

Severe reactive astrogliosis with compact glial scar formation also includes changes in gene expression, cellular
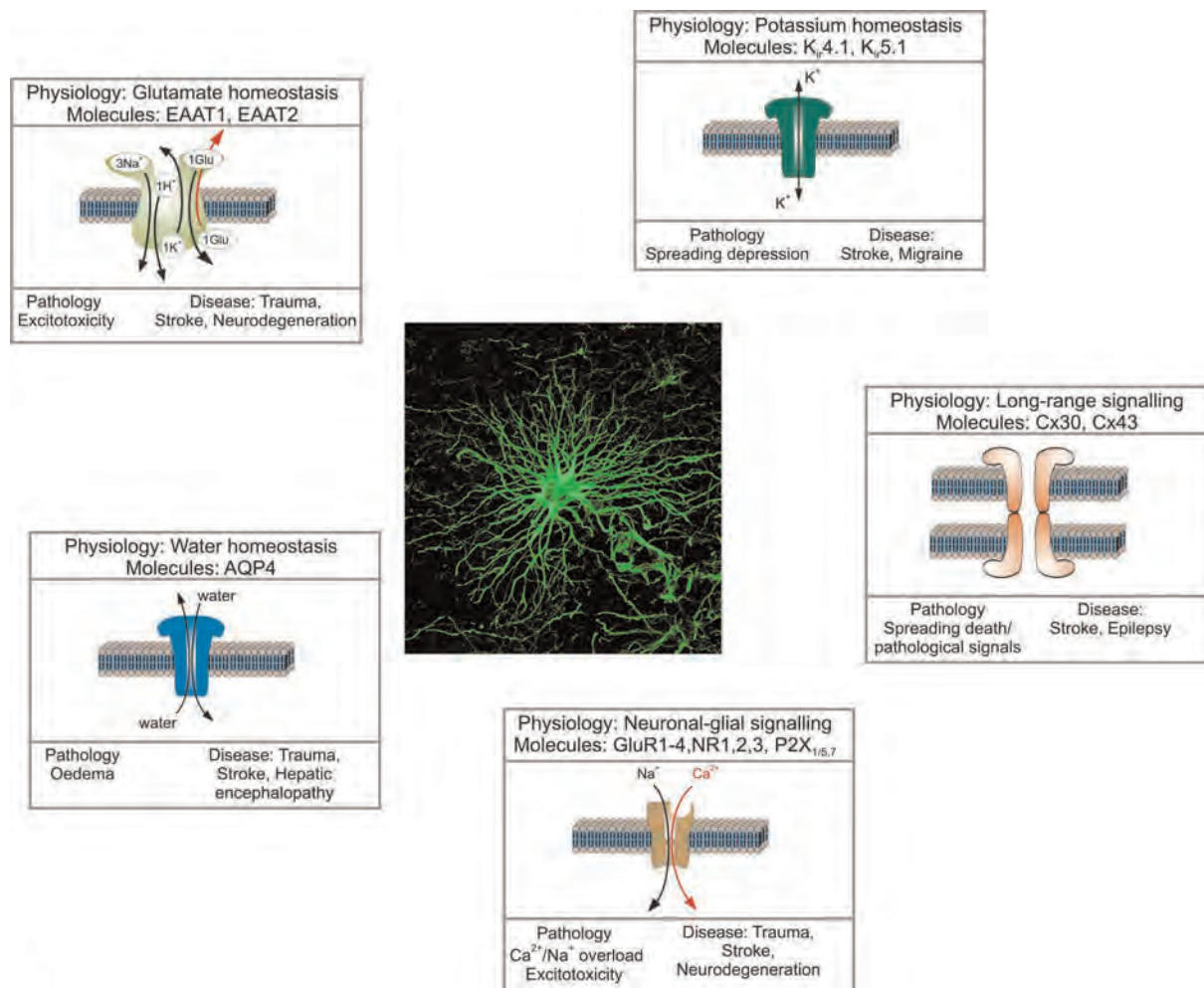
Figure 1   General pathophysiology of astroglia
The homoeostatic cascades expressed in astrocytes control various aspects of CNS homoeostasis including extracellular ion homoeostasis (K$^+$ buffering via K$_{ir}$ channels, Na/K pump and K transporters), regulate movements and distribution of water (via aquaporins and connexins), control extracellular concentration of neurotransmitters (by dedicated transporters) and provide the main reactive oxygen species scavenging system. In pathological conditions, the very same systems may contribute to brain damage. Failure in water transport triggers brain oedema, reversal of neurotransmitter transporters contributes to glutamate excitotoxicity, inadequate K$^+$ buffering promotes further overexcitation of neural cells and spreading depression, and connexins become a conduit for death signals.

hypertrophy and astrocyte proliferation, with pronounced overlapping of processes to form compact borders around areas of severe tissue damage, necrosis, infection or autoimmune-triggered inflammatory infiltration (Bush et al., 1999; Faulkner et al., 2004; Drogemuller et al., 2008; Herrmann et al., 2008; Voskuhl et al., 2009). These glial scars include other cell types, in particular fibromeningeal and other glial cells (Bundesen et al., 2003; Herrmann et al., 2008; Sofroniew, 2009; Sofroniew and Vinters, 2010), and deposit collagenous extracellular matrix that contains many molecular cues that inhibit axonal and cellular migration (Silver and Miller, 2004). Triggering insults include penetrating trauma, severe contusive trauma, invasive infections or abscess formation, neoplasm, chronic neurodegeneration, or systemically triggered inflammatory challenges. It is

noteworthy that the glial scar formation is associated with substantive tissue reorganization and structural changes that are long lasting and persist long after the triggering insult may have resolved.

The functional roles exerted by reactive astrocytes at different points along their continuum of potential responses to different kinds and severities of CNS insults are only beginning to be understood. Although astrogliosis and scar formation have often been viewed as maladaptive and purely detrimental responses, this is not the case. There are now many different kinds of evidence *in vivo* and *in vitro* that reactive astrocytes maintain their homeostatic mechanisms and can protect CNS cells and tissue in various ways, including by (i) uptake of potentially excitotoxic glutamate (Rothstein et al., 1996; Bush et al., 1999; Swanson et al.,
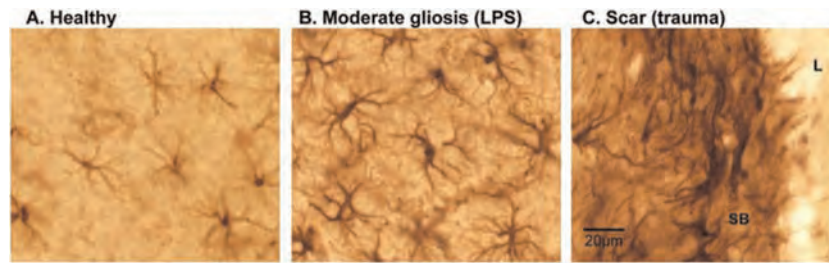
Figure 2 Appearance of astrocytes and different types of reactive astrocytes in mouse cerebral cortex
Images show immunohistochemistry for the intermediate filament protein, GFAP, which visualizes the cell cytoskeleton. (**A**) In healthy cortex, some, but not all astrocytes express detectable levels of GFAP; cell bodies are small and the cytoskeleton is thin and restricted largely to the proximal portions of cell processes. (**B**) In response to the bacterial antigen, lipopolysaccharide (LPS) injected into the lateral cerebral ventricle, cortical astrocytes become moderately reactive, with up-regulation of GFAP expression such that it is now detectable in all astrocytes. In addition, there is substantial hypertrophy of the astrocyte cell bodies as well as hypertrophy of stem processes and associated cytoskeleton. However, there is no astrocyte proliferation and individual cells continue to respect their individual, non-overlapping domains. (**C**) In response to a severe traumatic injury that creates a lesion (L) with tissue necrosis and invasion of inflammatory cells, astrocytes not only become reactive but also proliferate in the immediate vicinity of the lesion and form a scar with a dense scar border (SB) that comprises many newly generated astrocytes that do not exhibit individual domains and instead have many overlapping and intermingling processes. All images are at the same magnification. Scale bar=20 μm (Photos courtesy of the Sofroniew laboratory).

2004); (ii) protection from oxidative stress via glutathione production (Chen et al., 2001; Shih et al., 2003; Swanson et al., 2004; Vargas et al., 2008); (iii) protection via adenosine release that exerts systemic inhibitory and anti-inflammatory action (Lin et al., 2008); (iv) protection from NH_4^+ toxicity (Rao et al., 2005); (v) protection by degradation of Aβ (amyloid β-peptide; Koistinaho et al., 2004); (vi) facilitating blood–brain barrier repair (Bush et al., 1999); (vii) reducing vasogenic oedema after trauma, stroke or obstructive hydrocephalus (Bush et al., 1999; Zador et al. 2009); and (viii) stabilizing extracellular fluid and ion balance and reducing seizure threshold (Zador et al., 2009). Different types of transgenic models from different laboratories show that either ablation or attenuation of reactive astrogliosis causes increased lesion size, increased neuronal loss, demyelination and exacerbated loss of function after traumatic injury, stroke, autoimmune attack or infection (Bush et al., 1999; Faulkner et al. 2004; Myer et al., 2006; Okada et al., 2006; Drogemuller et al., 2008; Herrmann et al., 2008; Li et al., 2008; Voskuhl et al., 2009). Together, these findings indicate that reactive astrocytes exert essential neuroprotective functions. In addition, experimental evidence indicates that reactive astrocytes play important roles in regulating CNS inflammation, and that astrocyte scars act as essential functional barriers that restrict the migration of inflammatory cells and infectious agents and limit spread into adjacent healthy tissue (Sofroniew, 2005; Sofroniew, 2009; Sofroniew and Vinters, 2010).

THE GENETIC ASTROGLIOPATHOLOGY: ALEXANDER DISEASE

In considering how astrocyte dysfunction contributes to neurological disease, interpreting most models and natural diseases is complicated since there is presumed to be pre-existing injury to other cell types that then induces the changes in astrocytes. It is worth considering the simpler situation where astrocytes are clearly the primary instigator of disease – Alexander disease. This rare disorder arises from mutations in the major intermediate filament protein of astrocytes, GFAP (Brenner et al., 2001). Although GFAP is expressed at lower levels in a number of cell types throughout the body, many lines of evidence point to initial dysfunction of astrocytes as the key initiating event that then leads to a cascade of effects, ultimately affecting every other cell type in the CNS (Brenner et al., 2009).

Alexander disease is generally classified among the leucodystrophies because the initial descriptions primarily focused on younger onset patients who had dramatic white matter deficits, especially involving the frontal lobes. From its initial description (Alexander, 1949), the disease was associated with distinctive pathology involving accumulations of cytoplasmic protein aggregates within the cell bodies and processes of astrocytes, known as Rosenthal fibres, which contain GFAP and other proteins (Figure 3). The most common classification is based on age of onset (early, juvenile and adult; Li et al., 2005). The younger patients typically have seizures or any of several psychomotor delays as their initial symptoms, with forebrain predominance to their lesions. In contrast, the older patients typically present with gait disturbances, bulbar signs, or autonomic dysfunction, and have a hindbrain predominance of their lesions, sometimes including atrophy of the medulla and cervical spinal cord. All forms are typically progressive, with median survivals reported as 3.6 years for the early onset group and 8 years for the juvenile group (and undefined for adult) (Li et al., 2005). A more recent classification recommends division into just two groups, Types 1 and 2, based on particular
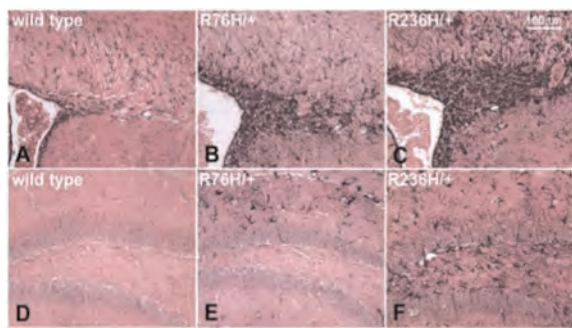
Figure 3 Immunostaining for GFAP in brain tissue from mouse models of Alexander disease showing abundant Rosenthal fibres in the periventricular region
GFAP immunohistochemistry in the periventricular white matter of (**A**) wild-type or (**B, C**) knock-in point mutants expressing either R76H or R236H mutant forms of GFAP (equivalent to the common R79H and R239H mutations in human GFAP). Abundant Rosenthal fibres with increased immunoreactivity for GFAP are particularly evident in periventricular and white matter astrocytes of adult mice (3 months old). Reproduced with permission from Figures 3(A)–3(C) of Hagemann et al. (2006) ©2006 Society for Neuroscience.

clustering of symptoms (Prust et al., 2011). In addition, survival may be longer for both groups than previously imagined.

As noted above, all forms of Alexander disease are now associated with mutations in GFAP (Li et al., 2005). Most of these mutations are heterozygous single base pair changes within the coding region that predict single amino acid changes in the translated protein. A small number of mutations involve in frame deletions or insertions. All are heterozygous, genetically dominant, with nearly 100% penetrance. A very small number of patients with presumed Alexander disease have no identifiable coding region mutations, but in theory a similar disease could arise from other types of alterations at the GFAP locus such as promoter mutations or gene duplication. Most mutations occur *de novo* (as expected when survival is short), although later onset patients can survive to reproductive age and transmit the mutations to subsequent generations with typical autosomal dominant patterns of inheritance. A recent population-based survey in Japan reported a prevalence of 1 in 2.7 million, but this value may be low because the study excluded severely affected infants who died at very young ages and adult-onset patients are often missed (Yoshida et al., 2011).

How do GFAP mutations cause disease? There are no clear answers to this question yet, partly a reflection of how little we understand the normal functions of GFAP and other intermediate filaments. It is interesting that the known disease-causing mutations (now numbering 71 out of a total 432 amino acid sequence) are sprinkled throughout the rod and tail domains (Brenner et al., 2009; http://www.waisman.wisc.edu/alexander/mutations). The surprising fact is that all of these mutations essentially cause the same disease. Genotype–phenotype correlations are still only speculative, due in large part to the paucity of patients with any particular mutation. While the R239H mutations

seem to consistently cause early onset disease, the R416W mutation has been found in all forms of the disease. Several examples exist of the same mutation causing different forms of the disease, even within the same family (Stumpf et al., 2003; Messing et al., 2011). It is possible that head domain mutations do occur, but are early lethal and so never reach the stage of diagnosis.

Unlike most of the other intermediate filament diseases, GFAP mutations appear to act in a gain-of-function fashion. In support of this hypothesis is the absence of truncation or null mutations among the human patients (Brenner et al., 2009), the dissimilarity in phenotype between mouse knockouts of GFAP and the human disease (Gomi et al., 1995; Pekny et al., 1995; McCall et al., 1996), and the finding that overexpression of wild-type protein alone to sufficient levels is lethal and replicates the key pathological feature of the disease, Rosenthal fibres (Messing et al., 1998). Using cell culture models, Tang and Goldman (2006) have found that forced overexpression of wild-type and mutant GFAP leads to pleiotropic effects, with activation of JNK (c-Jun N-terminal kinase) and p38 MLK (mixed-lineage kinase) stress pathways and impairment of the proteasome. One or more positive feedback loops then form to further enhance the toxic accumulation of GFAP. Paradoxically, there is also enhancement of autophagy, which occurs by both mTOR (mammalian target of rapamycin)-dependent and -independent mechanisms (Tang and Goldman, 2008). Mouse models show evidence of oxidative stress, particularly in white matter (Hagemann et al., 2006), and the cell culture models display increased sensitivity to camptothecin-induced apoptosis and H_2O_2-induced cell death (Tang and Goldman, 2006; Cho and Messing, 2009).

How GFAP mutations and/or excess activate these stress pathways and impact on the protein degradation machinery of the cell is not clear. Normally GFAP exists in an equilibrium between Triton-X soluble and insoluble pools, with the majority being insoluble. These two pools roughly correspond to the process by which monomers (which are soluble) gradually assemble into higher-ordered structures and finally mature intermediate filaments (which are insoluble). Expression of mutant GFAPs cause a distinct shift in this equilibrium towards the insoluble pool, perhaps reflecting the unknown proportion that accumulates in the Rosenthal fibres themselves (Hsiao et al., 2005). Interestingly, the shift towards insolubility is also displayed by the small stress protein αB-crystallin, which is a normal-binding partner of GFAP and accumulates in the Rosenthal fibres as well (Der Perng et al., 2006; Perng et al., 2008). Studies in mouse models have shown that αB-crystallin deficiency increases mortality (Hagemann et al., 2009). Perhaps αB-crystallin is sequestered to the point of deficiency? Hence, one hypothesis is that the Alexander phenotype reflects a combination of both gain of function (GFAP) and loss of function (αB-crystallin). One cannot blame the full Alexander phenotype on αB-crystallin deficiency alone; however, since mouse knockouts of αB-crystallin and humans with a nearly complete deletion at

this locus have predominantly skeletal muscle phenotypes, with no evidence yet for neurological consequences (Brady et al., 2001; Del Bigio et al., 2011).

Are the astrocytes in Alexander disease simply extreme examples of reactive astrocytes? They do share some properties considered fundamental to the gliotic phenotype, such as increased expression of GFAP and the endothelin B receptor (Hagemann et al., 2006). Recent studies point towards impaired expression of the glutamate transporter Glt-1 (Hagemann et al., 2009; Tian et al., 2010), perhaps analogous to what occurs in other neurodegenerative diseases such as ALS (amyotrophic lateral sclerosis). However, as (probably) noted above, reactive astrocytes are highly diverse and likely differ considerably depending on the nature of insult, location in the nervous system, and stage of disease (Hamby and Sofroniew, 2010). In the case of Alexander disease, the initial insult is mutant GFAP, which may produce toxic forms that are unique to this condition and cause types of astrocyte dysfunction different from those in other types of reactive astrocytes. Interestingly, the Goldman laboratory has identified toxic oligomers in the soluble pool of GFAP that may be responsible for the impairment in proteasome function noted above, and also showed that this effect could be mitigated by co-expression of the small stress protein, αB-crystallin (Tang et al., 2010). That αB-crystallin may be a key modifier of astrocyte function, and potential therapeutic target, is also indicated by its ability to rescue mouse models of Alexander disease from an otherwise lethal phenotype induced by GFAP mutations and excess (Hagemann et al., 2009).

Despite the many gaps in our understanding of the mechanisms and impact of astrocyte dysfunction in Alexander disease, several strategies for therapy have been suggested (Messing et al., 2010). The most obvious approach is to reduce the expression or accumulation of GFAP, so as to avoid the initial insult that drives the entire process. One drug screen has already been completed using wild-type astrocytes in primary culture (Cho et al., 2010), and similar drug screens using astrocytes expressing mutant protein are underway. A second approach is to target downstream effects of GFAP toxicity, such as the proposed change in Glt-1 expression. Ceftriaxone, already in clinical trials for ALS, may be a good candidate for such an approach (Rothstein et al., 2005). Finally, manipulation of stress pathways such as those regulating αB-crystallin may prove effective. New *Drosophila* models of GFAP toxicity in glia have just been developed that will prove especially valuable for dissecting the pathways to dysfunction, and may even be suitable for drug screening (Wang et al., 2011a).

THE ACUTE ASTROGLIOPATHOLOGY: ISCHAEMIA AND STROKE

Stroke remains a major source of death and disability in the United States as a stroke-induced death occurs every 3 min.

Over the past couple of decades, dozens of clinical trials tested neuroprotective agents in the treatment of stroke. These efforts largely targeted neuronal-specific cell death ignoring the roles of other cell types such as astrocytes, microglia and the vasculature. The failure of these trials has evoked a keen interest in elucidating the influence of non-neuronal cell types on brain survival and function during stroke. As discussed in detail at the beginning of this paper a large body of literature supports a pivotal influence of astrocytes on multiple processes within the brain, which impact the survival and function of neurons as well as the vasculature. Yet, astrocyte function may be both protective and harmful. Interestingly, the same molecule released by astrocytes may have opposing actions on brain tissue viability depending on their temporal expression following stroke. Determining the critical temporal windows and the molecular mechanisms by which astrocyte function either reduces or enhances injury is important to discern and will be a matter of the discussion to follow.

During the first few hours after the onset of ischaemia, the acute phase of stroke, several astrocyte functions may reduce ischaemic damage including the uptake of glutamate, scavenging of reactive oxygen species and uptake of K^+ (Chen and Swanson, 2003). Yet many of these functions are likely compromised in the inhospitable environment of the ischaemic brain. In the stroke core, where glucose and oxygen supply are greatly reduced, the severe loss of energy substrates can lead to astrocyte membrane depolarization and death. If the membrane potential of astrocytes is compromised too severely, glutamate flux may reverse leading to release of glutamate by astrocytes and neuronal excitotoxicity. Although astrocytes in culture are more tolerant to OGD (oxygen glucose deprivation) compared with neurons, they are particularly susceptible to damage during acidosis and when ion concentrations of Na^+ and Ca^{2+} are altered to model those found in the stroke core (Giffard et al., 1990, 2000; Chesler, 2005). In fact, some studies suggest that astrocytes may be more vulnerable to ischaemia than neurons (Garcia et al., 1993; Liu et al., 1999), although this point is debated (Gurer et al., 2009). Regardless, when astrocyte death occurs through necrosis, this unstructured loss of intracellular constituents into the extracellular space would be expected to enhance stroke damage by increasing inflammation and oedema.

Given that cell death in the stroke core occurs early and is overwhelming, research has focused on preventing delayed neuronal death in the stroke penumbra and reducing the growth of stroke volume in the hours and days after stroke onset. One clinical trial manipulated astrocyte function as a means to diminish the delayed growth of stroke volume. One of the mechanisms by which astrocytes likely enhance neuronal death following stroke is through the release of S100β (Matsui et al., 2002), which is toxic to neurons when present at high levels (Hu et al., 1997). AA (arundic acid; ONO-2506) was identified through a chemical screen as a compound that could diminish activation of astrocytes and S100β expression. In fact, AA reduced delayed

growth of infarct volume and improved functional outcome in rodents (Tateishi et al., 2002). The protective effect of AA was greater if administered 24 h after stroke onset compared with its administration immediately after stroke onset, suggesting that its window of efficacy is very much extended compared with most neuroprotectants. A multiple-centre trial testing the efficacy of AA to reduce stroke-related disability was conducted. However, this trial was terminated early for lack of efficacy. Yet, clinical studies suggest that growth of stroke volume occurs in a subset of patients (Sorensen et al., 1996; Beaulieu et al., 1999; Barrett et al., 2009). As such, this delayed growth of stroke volume may be a worthy target for future work if this subset of patients can be reliably identified and targeted.

In the days and weeks after stroke onset, astrocytes release a number of neuroprotectants including EPO (erythropoietin), VEGF (vascular endothelial growth factor) and glial-derived neurotrophic factor, all of which may either reduce ischaemic neuronal damage or improve functional recovery following stroke (Kitagawa et al., 1999; Zhang et al., 2000; Hermann et al., 2001; Harvey et al., 2003; Jelkmann and Wagner, 2004; Chavez et al., 2006). In particular, the neuroprotective properties of EPO have been studied (Masuda et al., 1994; Marti et al., 1997; Ruscher et al., 2002; Prass et al., 2003). For example, EPO reduces neuronal death with OGD (Ruscher et al., 2002), glutamate toxicity (Morishita et al., 1997), and nitric oxide induced death (Sakanaka et al., 1998). Conditioned media taken from hypoxic astrocyte cultures is protective to neurons exposed to OGD through the actions of EPO, demonstrating the importance of astrocytes in mediating neuroprotection under these circumstances (Chavez et al., 2006). Similar to the *in vitro* experiments, protective effects have been described for EPO *in vivo* during stroke (Bernaudin et al., 1999; Siren et al., 2001). Clinical trials were performed to establish the safety and efficacy of EPO in stroke treatment. An initial Phase II trial did not identify any safety concerns and suggested efficacy of EPO protection in acute stroke (Ehrenreich et al., 2002). A subsequent Phase III double-blinded, placebo-controlled trial enrolled 522 patients (Ehrenreich et al., 2009). Patients were treated with EPO within 6 h of the onset of their symptoms and clinical outcome was evaluated at 30 and 90 days after stroke (Ehrenreich et al., 2009). Unfortunately, EPO did not improve clinical outcome compared with the placebo. This negative result reminds us that all neuroprotective trials are hampered by the problem of delivery of a compound to a poorly perfused area of brain within a short time-frame after stroke onset. Yet, EPO could still be a potential agent to improve functional outcome after stroke. For example, EPO has multiple restorative functions, including promoting angiogenesis and neurogenesis (Shingo et al., 2001; Byts and Siren, 2009; Siren et al., 2009). To harness the restorative power of EPO in stroke, it is important to examine the mechanisms by which the temporal expression of endogenous EPO may be enhanced in astrocytes following stroke.

The transition from the acute stroke period to the sub-acute/chronic time frame after stroke can dramatically change the functional consequences of astrocyte-released proteins. Both VEGF and MMP-9 (matrix metalloproteinase-9) have dramatically different effects in the acute versus sub-acute time frames following onset of ischaemia. VEGF is expressed in astrocytes, as well as other cell types, during hypoxia and stroke (Sinor et al., 1998; Lee et al., 1999). While VEGF is neuroprotective in culture (Jin et al., 2000), administration of VEGF at the onset of stroke increases stroke volume (Jelkmann and Wagner, 2004). This pathological effect of VEGF is mediated by the ability of VEGF to enhance blood–brain barrier permeability, which leads to brain oedema. In contrast, when administered 2 days after stroke onset during the sub-acute stroke period, VEGF enhances angiogenesis and recovery from stroke (Jelkmann and Wagner, 2004). Thus, the protective or pathological actions of VEGF are determined by its temporal expression. Similarly, MMP-9 has pathological or adaptive functions depending on its temporal expression following stroke (Zhao et al., 2006). MMPs cleave extracellular matrix proteins, which are important in modulating cellular interactions during tissue remodelling in disease and trauma. It is well described that MMP-9 increases blood–brain barrier permeability and increases ischaemia-induced damage during acute stroke (Cunningham et al., 2005; Jian Liu and Rosenberg, 2005). In contrast, several days after stroke onset MMP-9 serves a neuroprotective role by cleaving and activating VEGF (Zhao et al., 2006). During this delayed time-frame, MMP is highly expressed on both astrocytes and neurons. In addition, VEGF localizes primarily to astrocytes, suggesting that astrocytes significantly contribute to this protective function of MMP-9. These temporally distinct and divergent actions of VEGF and MMP-9 demonstrate the important concept that astrocyte function may change from a pathological to adaptive depending on the temporal profile of expression following ischemia. As such, future therapeutics targeting these functions will need to carefully consider these critical time windows for intervention.

In the weeks to months following stroke onset, profound synaptic plasticity occurs in the peri-infarct cortex, which influences functional recovery (Carmichael, 2003; Nudo, 2007). Astrocytes have prominent roles in modifying synaptic plasticity and formation of new synapses (see above). Emerging evidence suggests that astrocytes release extracellular matrix proteins that encourage neurite outgrowth and plasticity and therefore may be a target for therapeutic intervention. For example, the TSP1/2 (thrombospondins 1 and 2), which are extracellular glycoproteins, are highly induced after stroke on astrocytes (Liauw et al., 2008). In cultures, the application of TSP1/2 enhances synaptogenesis several fold (Christopherson et al., 2005). Interestingly, in stroke models, synaptic number and axonal sprouting were reduced in transgenic knockout mice lacking TSP1/2 function (Liauw et al., 2008). Recovery of functional tests was also diminished in these TSP1/2 KO mice. Based on these results,

designing treatments that increase TSP1/2 function or astrocyte release of TSP 1/2 may enhance stroke recovery.

Similar to the above discussions, the effect of reactive astrocytosis is also dependent on its time course after acute head trauma. The role of reactive astrocytes has traditionally been viewed as a process leading to glial scar formation, reducing the ability of the brain to form new synaptic connections and axon outgrowth. Yet, work over the last several years has illustrated that reactive astrocytosis contributes to tissue survival in several conditions, which is in part dictated by the time course after injury in which it occurs. One tool for examining reactive astrocytes is by utilizing transgenic mice with loss of GFAP and vimentin (Pekny and Nilsson, 2005). These mice have diminished reactive astrocytosis. Interestingly, the effect of reactive astrocytosis changes temporally after injury. For example, after neurotrauma, $GFAP^{-/-}$:Vimentin$^{-/-}$ mice have increased loss of synapses during the acute phase of the injury, but synaptic number is enhanced in the weeks following acute injury (Wilhelmsson et al., 2004). In stroke, $GFAP^{-/-}$:Vimentin$^{-/-}$ mice have increased stroke volume, suggesting a protective role under these conditions (Li et al., 2008). Similarly, ablation of proliferating reactive astrocytes enhanced spinal cord injury following spinal cord trauma (Faulkner et al., 2004) and traumatic brain injury (Myer et al., 2006), while it also enhanced neurite outgrowth (Bush et al., 1999).

THE CHRONIC ASTROGLIOPATHOLOGY: EPILEPSY

Epilepsy or the occurrence of recurrent seizures is a behavioural syndrome, that is, a component of several neurological conditions. Several brain foci associated with seizure generation are populated by increased numbers of astrocytes. Such foci include hippocampal seizure foci in temporal lobe epilepsy, several types of mass lesions in the brain (low-grade astrocytomas, oligodendrogliomas and arteriovenous malformations) and tuberous sclerosis. Gliotic scar formation is a prominent feature of human epilepsy (Foerster and Penfield, 1930; Penfield and Humphreys, 1940). What is the role of astrocytes in epileptogenesis in these neurological conditions?

Epileptogenesis literally means the beginning of epilepsy or spontaneous recurrent seizures. Do astrocytes play a role in the mechanisms necessary for spontaneous seizures to first appear or do astrocytes play a role in seizure maintenance in the chronic stages of epilepsy? There is some evidence to support a primary role of astrocytes in seizure generation, particularly in animal models. In the EL (epileptic) mouse the hippocampus is important for the generation of behavioural seizures. However, there is no neuronal injury or loss in this model but an increase in expression of astrocytes around the

age when seizures appear (Drage et al., 2002), and these astrocytes have reduced glutamate transporters, suggesting a primary role for astrocytes, perhaps through defective glutamate clearance at the seizure focus (Ingram et al., 2001). More direct evidence is found mice in which there is astrocyte-specific inactivation of the Tsc1 gene (Tsc1 cKO mice). In these mice, there is an increase in proliferating GFAP-immunoreactive astrocytes throughout the brain (Uhlmann et al., 2002), with the most distinctive histological alterations seen in the hippocampus. What is significant is that these astrocyte-specific cKO mutant mice did not show cortical tubers or defects in neocortical lamination. These mice have electrographically confirmed seizures shortly after astrocyte proliferation has begun (Uhlmann et al., 2002). Further, the astrocytes in Tsc1 cKO exhibit decreased expression of the glutamate transporters GLT-1 and GLAST and a functional decrease in glutamate transport current in astrocytes in hippocampal slices and astrocyte cultures (Wong et al., 2003). Such changes in glutamate transporters may lead to the extracellular accumulation of glutamate, which could cause hyperexcitability of neurons and seizures. Cultured Tsc1-deficient astrocytes and hippocampal slices from cKO mice also exhibited reduced Kir currents and decreased expression of specific K_{ir} channel protein subunits Kir2.1 and Kir6.1. Thus impaired extracellular K^+ uptake by astrocytes may also contribute to neuronal hyperexcitability and epileptogenesis in this Tsc1 cKO mouse model (Jansen et al., 2005).

Much of our understanding of the role of glia in human epilepsy is obtained from the study of seizure foci surgically removed for the control of medically intractable seizures (de Lanerolle et al., 2010). The one limitation in studying human tissue that it is taken from patients is that they have had seizures for a considerable period (6–20 years) prior to surgery. Thus it is more difficult to draw conclusions from this patient group on the role of astrocytes in the early (acute) stages of epileptogenesis.

The seizure focus that has received the most study is the hippocampus from patients with medically intractable temporal lobe epilepsy. The examination of such hippocampi indicates that approximately 40–65% of these hippocampi have hippocampal sclerosis. Eighty percent of these sclerotic patients have an excellent surgical outcome. The sclerotic hippocampi have a very high density of astrocytes and these astrocytes have many distinctive properties compared with astrocytes from non-sclerotic hippocampi. Differences in these astrocytes are seen in their cell membrane properties – they show increased expression of the glutamate receptors mGluR2/3 (metabotropic glutamate receptor 2/3); mGluR4, mGluR8 and GluR1 receptors that have an elevated ratio of flip-to-flop mRNA splice variants (Seifert et al., 2002, 2004). The expression of membrane transporter molecules is also altered. Prominent among these are aquaporin 4 molecules, where their polarity of distribution on the astrocytes is altered with reduced expression on the perivascular end feet and unchanged on the

membrane facing the neuropil. The GABA (γ-aminobutyric acid) transporter GAT-3 (GABA transporter 3) expression is increased on protoplasmic astrocytes in regions of relative neuronal sparing such as dentate gyrus and hilus. There is some disagreement in the literature as to whether the glutamate transporters EAAT1 (excitatory amino acid transporter 1) and EAAT2 are also reduced. The membrane Na$^+$ channels and α1C subunit of the calcium ion channels are also up-regulated, suggesting that astrocytes in sclerotic hippocampi have a significant change in their membrane current characteristics. The inwardly rectifying potassium ion (K_{ir}4.1) channels are also shown to be impaired, significantly impeding removal of K$^+$ ions from the extracellular space (Bordey and Sontheimer, 1998; Hinterkeuser et al., 2000; Schroder et al., 2000). Among the astrocyte specific enzymes, glutamine synthetase activity is reduced (Eid et al., 2004), impeding glutamate clearance and thus leading to increases in extracellular glutamate levels (During and Spencer, 1993). There is also a reduced capacity for glutamine synthesis and ammonia detoxification. Levels of other astrocyte specific enzymes such as GDH (glutamate dehydrogenase), aspartate aminotransferase and lactate dehydrogenase (Malthankar-Phatak et al., 2006) are also altered in astrocytes in sclerotic hippocampi.

Gene expression studies in sclerotic hippocampi have also suggested changes in the expression of several genes associated with astrocytes. Among those up-regulated are those involved with immune and inflammatory functions, including several chemokines and cytokines, class II MHC antigen genes and interleukins and complement factors (Aronica et al., 2007; Lee et al., 2007). Several molecules associated with the astrocyte/microvascular interface are also altered, in particular increases in EPO-r (EPO receptor), the MDR1 (multidrug resistance gene-1) encoded P-glycoprotein (Tishler et al., 1995), CD44 and plectin 1 (Lee et al., 2007), among others (de Lanerolle et al., 2010).

Associated with the above molecular anatomical changes in astrocytes in sclerotic hippocampi are also changes in their function. Some astrocytes in primary cultures derived from sclerotic hippocampi and *in vitro* hippocampal slices are capable of generating action potential-like responses in response to depolarizing currents (Bordey and Sontheimer, 1998; O'Connor et al., 1998). Astrocytes from sclerotic hippocampi respond to glutamate with elevated intracellular Ca^{2+} release and Ca^{2+} oscillations and waves (Lee et al., 1995). Additionally, several lines of evidence suggest that the altered properties of sclerotic astrocytes, particularly down-regulation of glutamine synthetase, also alter glutamine–glutamate cycling in hippocampal seizure foci resulting in increased extracellular glutamate levels before and during seizures (Figure 4).

More recent studies suggest the recognition of two functional classes of astrocytes. One type, sometimes referred to as GluR cells, is weakly positive for GFAP, expresses AMPA-type glutamate receptors and properties akin to NG2 cells. These cells are excitable (O'Connor et al., 1998). A second type of cell, the GluT cell, is more strongly GFAP positive, is more fibrous in appearance and expresses K$^+$ channels, but lack glutamate receptors (Matthias et al., 2003). Cells similar to GluR and GluT cells have been recognized in the human hippocampus, and though both types are found in normal hippocampi, an almost complete loss GluT cells is reported in sclerotic hippocampi (Hinterkeuser et al., 2000). It is most likely that it is these cells that have impaired K_{ir} channels. Further, the GluR cells in sclerotic hippocampus have increased levels of the Flip isoform of GluR1 receptor, suggesting an increased potential for excitability.
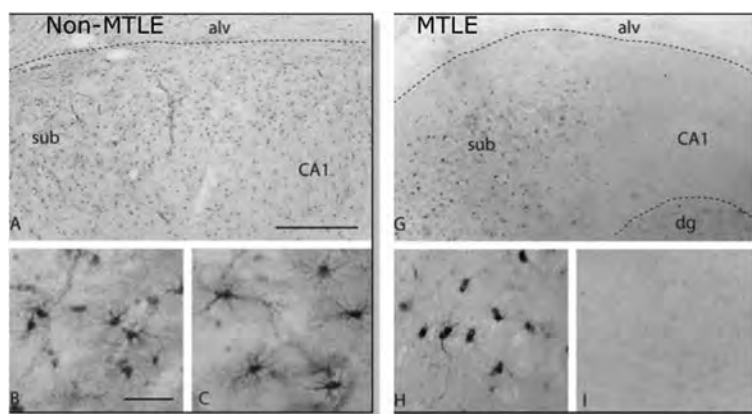

Figure 4   Expression of glutamine synthetase immunoreactivity in a non–sclerotic and sclerotic hippocampus
Glutamine synthetase immunoreactivty in the subiculum/CA1 region in a non-sclerotic [non–MTLE (mesial temporal lobe epilepsy)] (**A**) and sclerotic (MTLE) (**G**) hippocampus. Neurologically normal autopsy hippocampus shows a pattern of staining exactly similar to (**A**). (**B**, **C**) in higher magnification shows GS immunopositive astrocytes in both the subiculum and CA1 area of a normal or non-sclerotic hippocampus. The sclerotic hippocampus, in which the subiculum does not have neuronal loss shows GS immunoreactive astrocytes (**H**), whereas the neuron-depleted astrocyte-rich CA1 area (**I**) shows depletion of GS in astrocytes. (**A** and **G**) Scale bar=0.5 mm. (**B, C, H** and **I**) Scale bar=100 µm. Reprinted from The Lancet, 363, Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, Kim JH, Danbolt NC, Ottersren OP, de Lanerolle NC, Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy, 28-37, Copyright (2004), with permission from Elsevier.

What role do astrocytes play in a mature hippocampal seizure focus? As the above review suggests, they may play several roles. (i) Astrocytes may contribute to the high glutamate levels at seizure foci through defective glutamate clearance, and additionally active release of glutamate from GluR (NG2)-like cells due to enhanced intracellular Ca^{2+} release or by astrocyte swelling due to reduced aquaporin 4 transporters on perivascular end feet (de Lanerolle et al., 2010). These elevated glutamate levels may activate neurons in surrounding or adjacent undamaged regions such as the subiculum to generate seizure activity. (ii) Defective astrocytes may contribute increased extracellular potassium in the seizure focus. Impaired inwardly rectifying K^+ channels and decreased water flux due to reduced AQP transporters in astrocytic end feet may be contributory factors. (iii) The presence of excitable GluR or NG2-like cells with more glutamate-sensitive GluR1 receptors in the sclerotic seizure focus may directly contribute to an excitable focus. (iv) Astrocytes may also modulate the microvasculature, leading to vascular permeability and promoting entry of substances such as albumin or circulating leucocytes into the brain parenchyma with consequent seizure promoting effects (de Lanerolle et al., 2010). (v) The release of inflammatory and immune factors by astrocytes may also contribute to the development of the seizure focus in ways that are only just beginning to be understood.

Another interesting aspect of epilepsy is the destruction and loss of astrocytic domain organization (Figure 5). Several groups have shown that in the normal brain cortical and hippocampal astrocytes are organized in non-overlapping spatial domains with limited interdigitation of processes of adjacent cells (Bushong et al., 2002; Ogata and Kosaka, 2002; Halassa et al., 2007a, 2007b). Through a process termed 'tiling,' astrocytic processes grow within exclusive terri-

tories during development when neuronal and vascular territories are also being established. In the rodent brain, one astrocytic domain encompasses ~100000 synapses, whereas this number rises to 2000000 synapses in the brain of homo sapiens (Bushong et al., 2002; Oberheim et al., 2006). Each domain represents an area of the neuropil that is under control of a single astrocyte, being also an entity of synaptic modulation that is independent of neural networking. All synapses within one territory will be contacted by processes from only one single astrocyte. Reactive astrocytes in three very different murine models of epilepsy (post-traumatic injury, genetic susceptibility and systemic kainate exposure) all were associated with a 10-15-fold increase in overlap of processes of neighbouring astrocytes (Oberheim et al., 2008). A similar loss of astrocytic domain organization was noted in tissue surgically resected from patients resilient to medical treatment. It is important to note that astroglial domain organization was preserved in APP transgenic mice expressing a mutant variant of human amyloid precursor protein despite a striking up-regulation in GFAP expression. Thus, while the functional consequences of loss of astrocytic territories have not been established, it appears to be specifically linked to epilepsy. It is tempting to speculate that synapses receiving input from more than one astrocyte may not function optimally.

Are these changes in astrocytes in the hippocampal seizure focus secondary mechanisms in seizure development or are they causative? Comparison of these observations with animal studies discussed above, where astrocytes appear to have a more primary role in epilepsy, show that at least some of the astrocytic changes in the human focus may be causative, in particular impairment of K_{ir} channels and decreased expression of astrocytic glutamate transporters.
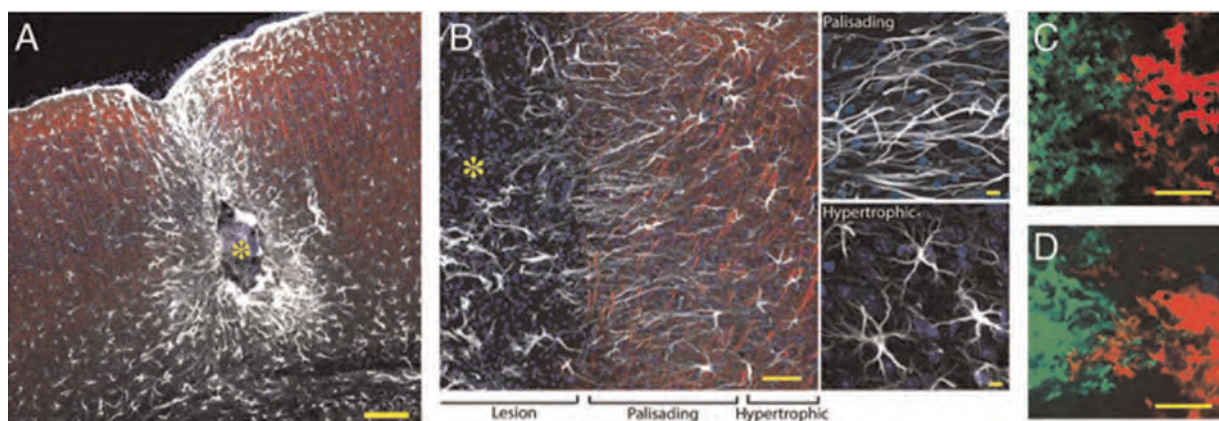
Figure 5   **Organization of reactive astrocytes in a model of post–traumatic epilepsy induced by cortical injection of a ferrous chloride solution**
(**A**) Site of cortical injury 6 months after injury. The centre of the lesion (yellow asterisk) is surrounded by palisading astrocytes and, at a greater distance, by hypertrophic astrocytes. The mouse exhibited daily multiple generalized grand mall seizures. (**B**) Higher power image of a similar lesion displaying palisading and hyperthrophic astrocytes. White, GFAP; red, Map2; blue, Sytox. (**C**, **D**) Neighbouring astrocytes in control, non-EL brain exhibit little overlap of processes (**C**), whereas extensive overlap of processes between two adjacent astrocytes is evident in a mouse with epilepsy (**D**). Neighbouring astrocytes were duolistically labelled with DiL (green) or DiD (ref). Scale bar=100 μm (**A**), 50 μm and 10 μm (**B**), 10 μm (**C**, **D**). See Oberheim et al. (2008) for details.

THE CHRONIC ASTROGLIOPATHOLOGY: NEURODEGENERATIVE DISEASES

Neurocentric views dominate our current understanding of neurodegeneration, which is generally defined as a process of neuronal death that underlie specific neurological deficits. At the same time it becomes clear that idiosyncratic lesions associated with different forms of neurodegenerative diseases (such as for example senile plaques or Lewy bodies) appear at the late stages of neurodegenerative pathologies and massive neuronal demise signals terminal stages of the disease. Our knowledge about early stages of neurodegenerative processes (when arguably the disease can be either halted or slowed down) is remarkably limited, and yet data accumulate suggesting that neurodegeneration begins from failures in brain homoeostasis and alterations in connectivity of neural networks that signals early cognitive impairments (Terry, 2000; Kano and Hashimoto, 2009; Nedergaard et al., 2010; Heneka et al., 2010). The many levels of brain homoeostasis (cellular, micro-architectural, vascular, metabolic, neuro-transmitter, ion, etc.) are controlled almost solely by neuroglia; and it is neuroglia that mount brain defence. With this in mind the progression of majority (if not all) of CNS disorders are determined by the ability of neuroglia to keep brain homoeostasis in stressed conditions, and the failure of glia to maintain homoeostatic balance signals irreparable damage and ultimate death of the neural tissue (Giaume et al., 2007). The pathological potential of astroglia in neurodegeneration is explored only superficially and yet (as we shall overview below) they seem to be involved in both early and late stages of many neurodegenerative diseases.

Astroglia in AD (Alzheimer's disease)

The pathological potential of neuroglia in *dementia praecox* was for the first time recognized by Alois Alzheimer, who found that glia populated senile plaques and closely contacted damaged neurones (Alzheimer, 1910). Conceptually there are two types of astroglial reactions observed in AD-affected brain tissues (Figure 6). Astroglial hypertrophy associated with increased GFAP and S100β levels, all indicative of generalized astrogliosis, are often observed in the post-mortem human tissues (Beach and McGeer, 1988; Griffin et al., 1989; Nagele et al., 2004; Mrak and Griffin, 2005), and similarly astrogliosis is detected in various AD animal models (Nagele et al., 2003; Rodriguez et al., 2009; Heneka et al., 2010). There is a degree of correlation between severity of astrogliosis and cognitive decline, although reactive astrocytes are not always associated with senile plaques (Simpson et al., 2010). The levels of GFAP alone cannot be predictable as little difference in GFAP expression was found in brains from non-demented and demented patients (Wharton et al., 2009). The second reaction of astroglia to AD progression was found recently in an animal model of familial AD, where reaction is manifest in the
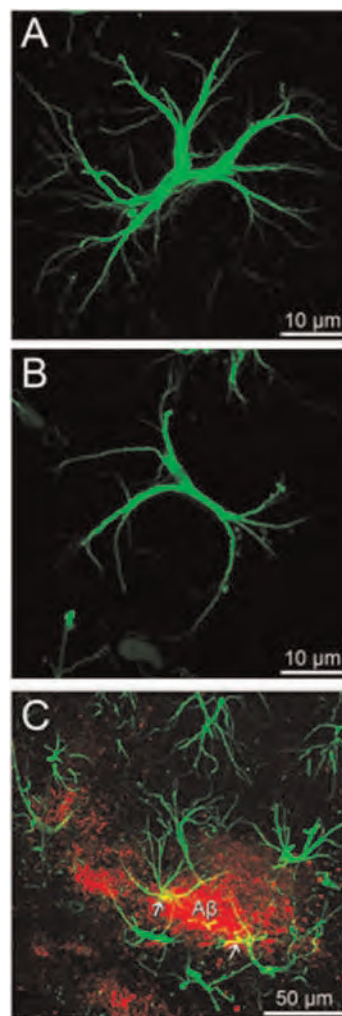
Figure 6 Astrocytes in neurodegeneration
(A) Fluorescence micrographs illustrating a normal hippocampal astrocyte labelled with anti-GFAP antibody with elongated and multiple radial processes in an old (18 months) control animal. (B) In age-matched $3 \times$ TG-AD animals, astrocytes show a morphological atrophy with a significant reduction in cell soma volume and area as well as a reduction in the number and width of processes. (C) Confocal image showing hypertrophic astrocytes (green) concentrated around Aβ plaques (red); occasionally some of the astrocytes show intracellular Aβ accumulation (yellow). Scale bar (A and B): 10 μm; (C): 50 μm.

generalized decrease of morphological presence of astrocytes signalling astroglial atrophy/degeneration. The atrophic changes in astrocytes were detected in several brain regions, including hippocampus, pre-frontal and entorhinal cortex (Olabarria et al., 2010; Kulijewicz-Nawrot et al., 2011; Olabarria et al., 2011; Yeh et al., 2011). The astroglial atrophy preceded the appearance of senile plaques and appeared first (as early as 1-month-old animals) in the entorhinal cortex, the region earliest affected by the AD pathology. Atrophic changes in astroglia were also observed in the neocortices from the post-mortem demented human brains (Senitz et al., 1995).

How do these two concomitant processes may affect homoeostatic/neuroprotective abilities of astrocytes? The answer to this question remains virtually unknown; for example several contradictory reports indicate that astrocytes in AD brain can participate in both clearance and degradation of Aβ (Guenette, 2003; Nicoll and Weller, 2003) as well as in the production of the latter (Heneka et al., 2005; Rossner et al., 2005). Astroglial accumulation and degradation of Aβ was shown *in vitro* (Wyss-Coray et al., 2003) and was observed in post-mortem entorhinal preparations (Nagele et al., 2003). Furthermore, it was found that reactive astrocytes from AD model mice (Tg2576) express the amyloid-degrading enzyme neprilysin (Apelt et al., 2003). Astroglial accumulation of Aβ, however, was very rarely found in $3 \times$ TG-AD mice (Rodriguez et al., 2009; Olabarria et al., 2010). At the same time astroglial production of Aβ was also reported: it appeared that, in conditions of chronic stress or amyloid load, reactive astrocytes begin to express γ-secretase (which normally is expressed only in neurons) and thus contribute to Aβ production (Hartlage-Rubsamen et al., 2003; Heneka et al., 2005; Rossner et al., 2005). The Aβ was additionally found to remodel astroglial metabolism, although the data remain similarly controversial: both increased (Allaman et al., 2010) and decreased (Soucek et al., 2003; Schubert et al., 2009) glucose utilization by Aβ-treated astrocytes were reported. This polymorphism of pathological astroglial phenotypes most likely results from multifaceted glial reaction to AD-like pathology which includes atrophic and astrogliotic changes.

Astrocytes in ALS

Clinical symptoms of ALS result from rapid progressive degeneration of motor neurons in the cortex, in the brain stem and in the spinal cord. Neuroglial reactions are prominent in ALS and most interestingly the early changes (observed in the human SOD1^{G93A} transgenic mouse model) are represented by astroglial degeneration and atrophy which preceded both neuronal death and clinical manifestation (Rossi et al., 2008; Rossi and Volterra, 2009). Astrocytes selectively expressing hSOD1 demonstrated glutamate excitotoxicity, and inhibition of SOD1 expression selectively in astrocytes retarded the ALS progression in transgenic animals (Yamanaka et al., 2008). At the late disease stages, prominent astrogliosis is observed, although atrophic astrocytes still can be visualized (McGeer and McGeer, 2002; Rossi et al., 2008).

Astrocytes in Parkinson's disease

The pathological role of astrocytes in Parkinson's disease is unknown; the late stages of the disease are characterized by reactive astrogliosis (McGeer and McGeer, 2008; Mena and Garcia de Yebenes, 2008). The density of astrocytes in the substantia nigra, which is primarily affected by PD pathology, is the lowest in the brain (Mena and Garcia de Yebenes, 2008). This may explain specific vulnerability of substantia nigra neurons to stress factors; in addition, astroglial cells are known to protect dopaminergic neurones *in vitro* and are instrumental for neuronal utilization of L-DOPA (Mena et al., 1996, 1999; Mena and Garcia de Yebenes, 2008).

Astrocytes in Wernicke encephalopathy

Wernicke encephalopathy, most likely caused by thiamine deficiency which triggers thalamo-cortical lesions resulting in ataxia, ophthalmoplegia and mental changes, is an example of specific astrodegeneration. Indeed the leading mechanism for the cause of severe excitotoxicity and neuronal death in Wernicke encephalopathy is the dramatic (up to 70%) decrease in expression of astroglial glutamate transporters EAAT1 and EAAT2 that compromises glutamate uptake. The decrease in EAAT1/EAAT2 was detected in human post-mortem tissues and in the rat thiamine-deficiency model of the disease (Hazell, 2009; Hazell et al., 2009). The signs of astroglial atrophy observed in Wernicke encephalopathy also include significant decrease in expression of GFAP, of glutamine synthetase and astroglial GAT-3 (Hazell, 2009; Hazell et al., 2009).

Astrocytes in non–AD dementia

Astroglial degeneration is also observed in various forms of non-AD neurodegeneration/dementia. Significant astro-degeneration and astroglial apoptosis was observed in early stages of fronto-temporal dementia; the degree of astro-degeneration was reported to correlate with the severity of dementia (Broe et al., 2004). In addition to these atrophic changes, profound astrogliosis was detected in post-mortem tissues form patients with fronto-temporal dementia (Kersaitis et al., 2004). In thalamic dementia astrogliosis was suggested to lead to neuronal death (Potts and Leech, 2005). HIV-1-associated dementia is also characterized by astro-gliosis and astrodegeneration; the degree of astroglial loss was reported to correlate with cognitive deficit (Thompson et al., 2001; Vanzani et al., 2006).

Astrodegeneration and astrogliosis drive neurodegeneration?

Analysis of the recent literature indicates the existence of multifaceted astroglial reactions in the progression of various neurodegenerative processes. Almost invariably these reactions are represented by astrodegeneration/astroglial atrophy and reactive astrogliosis. Balance between these two processes can determine the progression and outcome of the disease, thus placing astrocytes at the very core of neurodegenerative pathology (Rodriguez et al., 2009; Heneka et al., 2010; Verkhratsky et al., 2010; Rodriguez and Verkhratsky, 2011). Early astrodegeneration, for example, may

be responsible for initial cognitive impairments that accompany early stages of neurodegenerative diseases. It is well demonstrated that the earliest morphological symptoms of AD are represented by synaptic loss and the extent of this loss correlates with the degree of dementia (DeKosky and Scheff, 1990; Terry et al., 1991; Samuel et al., 1994; Coleman et al., 2004). Astroglial atrophy may indeed be the mechanism of early synaptic failure in neurodegeneration: reduced astroglial coverage impacts upon synaptic metabolic support, synaptic maintenance and neurotransmitter homoeostasis and turnover. Furthermore, astroglial degeneration affects brain homoeostasis and favours excitotoxicity. At the more advanced stages of neurodegeneration appearance of specific lesions triggers reactive astrogliosis and microglial activation, which acting in concert mount neuroinflammatory responses that ultimately lead to neuronal death and brain atrophy. Would this hypothesis survive the scrutiny of experimental test? Only specifically designed research can provide an answer.

CONCLUSIONS: POTENTIAL PATHOLOGICAL CONSEQUENCES OF ASTROCYTE LOSS OR GAIN OF FUNCTIONS

Based on the many different essential roles played by astrocytes in the healthy CNS, it appears likely that the loss of normal functions or gain of abnormal effects by astrocytes has the potential to lead to neuronal dysfunction or degeneration. In this regard, the potential for genetic polymorphisms in different individuals to influence astrocyte functions and dysfunctions may be of clinical interest. Both experimental and clinical examples now exist of how loss of astrocyte functions can precipitate neuronal dysfunction or degeneration. Transgenic mouse models show that deletion of genes selectively from astrocytes can lead to non-cell-autonomous neuronal dysfunction and degeneration. For example, selective deletion from astrocytes of either the endoribonuclease, Dicer, or of the Wnt-signalling pathway gene, APC (adenomatous poliposis coli), in both cases leads to cell-non-autonomous neuronal degeneration of cerebellar Purkinje neurons and in the case of Dicer, also of cerebellar granule neurons (Tao et al., 2011; Wang et al., 2011a). Similarly, the loss of function associated with astroglial atrophy can assume pathological relevance in synaptic weakening and decreased neuronal support in various forms of neurodegenerative pathology as discussed above. In addition, gain-of-function transgenic models indicate that selective targeting to astrocytes of a mutant form of the SOD (superoxide dismutase) associated with ALS leads to neuronal degeneration (Lobsiger and Cleveland, 2007; Nagai et al., 2007; Yamanaka et al., 2008). As the first recognized clinical example of an astrocyte genetic disorder, mutations in the astrocyte protein, GFAP, have been identified as the cause of

neuronal dysfunctions, including seizures, in the human clinical syndrome known as Alexander disease. The prominent association of reactive astrocytes with essentially all CNS insults has the potential for the functions or dysfunctions of these cells to influence CNS pathologies. Combination of mouse models of transgenic astrocyte manipulations with experimental models of CNS injury or disease show that genetic modulations of reactive astrogliosis and scar formation can markedly alter tissue repair, disease progression and functional outcome, such that ablation of astrocytes and attenuation of certain astrocyte functions exacerbates disease progression and tissue degeneration and worsens functional outcome (Bush et al., 1999; Faulkner et al., 2004; Myer et al., 2006; Drogemuller et al., 2008; Herrmann et al., 2008, Li et al., 2008; Voskuhl et al., 2009), whereas deletion of certain astrocyte genes appears to improve outcome in some situations (Brambilla et al., 2005, 2009; Okada et al., 2006). It is also important to acknowledge that human astrocytes are large, complex, and likely terminally differentiated cells. Astrocytes in rodent brains are several-fold smaller and maintain the potential for dividing. The much longer lifespan of humans combined with the more complex population of astrocytes, suggest that human astrocytes may participate to an even greater extent to disease progression than observations in rodent models suggest. Collectively, multiple findings point towards an enormous, yet incompletely understood, potential for astrocytes to contribute to, or play primary roles in, disease processes, tissue repair and functional outcome in a wide variety of clinical conditions (Sofroniew and Vinters, 2010), including stroke, epilepsy and neurodegenerative diseases.

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Critical role of aquaporin-4 (AQP4) in astrocytic Ca^{2+} signaling events elicited by cerebral edema

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Aquaporin-4 (AQP4) is a primary influx route for water during brain edema formation. Here, we provide evidence that brain swelling triggers Ca^{2+} signaling in astrocytes and that deletion of the Aqp4 gene markedly interferes with these events. Using in vivo two-photon imaging, we show that hypoosmotic stress (20% reduction in osmolarity) initiates astrocytic Ca^{2+} spikes and that deletion of Aqp4 reduces these signals. The Ca^{2+} signals are partly dependent on activation of P2 purinergic receptors, which was judged from the effects of appropriate antagonists applied to cortical slices. Supporting the involvement of purinergic signaling, osmotic stress was found to induce ATP release from cultured astrocytes in an AQP4-dependent manner. Our results suggest that AQP4 not only serves as an influx route for water but also is critical for initiating downstream signaling events that may affect and potentially exacerbate the pathological outcome in clinical conditions associated with brain edema.

endfeet | glial | two-photon

Using in vivo two-photon imaging of mice subjected to hypoosmotic stress, we have shown previously that astrocytes rapidly swell in the initial phase of edema development (1). These data are consistent with a number of studies suggesting that aquaporin-4 (AQP4) serves as a primary influx route for water from blood to brain (2, 3). AQP4 is strongly expressed in astrocytic endfeet (4), which form a continuous pericapillary sheath that is interrupted only by a narrow extracellular space (5).

In vitro studies clearly show that swelling of astrocytes leads to the activation of a number of signaling cascades (6, 7). Because astrocytes are prone to swell in experimental conditions associated with edema formation (8), this raises the question of whether the same signaling cascades are activated in early edema formation in vivo and whether they affect the clinical outcome.

Hypoosmotic stress induces brain edema with early accumulation of water in astrocytes (1, 9, 10). Thus, hypoosmotic stress provides a suitable experimental model to explore potential signaling mechanisms initiated by astrocytic swelling in vivo. Here, we use optical imaging to show that hypoosmotic stress induces Ca^{2+} spikes in astrocytes in vivo and that these spikes are potentiated in the presence of AQP4. We also provide in vitro data indicating that the AQP4-dependent Ca^{2+} signals are mediated in part by autocrine purinergic signaling. Our findings show that brain edema formation should not be seen merely as a process of passive water accumulation in brain but as a condition that sets in motion specific signaling processes that may significantly affect disease progression and morbidity.

Results

Aqp4 Deletion Reduces Swelling of Cortical Astrocytes Exposed to Mild Hypoosmotic Stress.
Immunofluorescence and Western blots confirmed the efficacy of the Aqp4$^{-/-}$ KO strategy (Fig. 1 A and B). Light microscopy revealed normal cytoarchitecture of cortex in Aqp4$^{-/-}$ mice. Specifically, astrocytes, visualized by GFAP immunolabeling, displayed normal morphology and intact endfeet (Fig. 1B).

To further validate the Aqp4 deletion, we performed volumetric analysis of astrocytic somata in acute cortical slices exposed to solution of reduced osmolarity (Fig. 1C). Astrocytes were readily detected by two-photon imaging after the slice had been incubated with the fluorescent dye Texas red hydrazide, an approach similar to that described for sulphorhodamine 101 (11, 12). Dye loading of slices obtained from transgenic mice that express EGFP under control of the Glt-1 promoter (Glt-1–EGFP BAC transgenic mice) (13) confirmed that Texas red hydrazide was selectively taken up by GFP-expressing astrocytes (Fig. 1D).

When exposed to 20% reduction in osmolarity, WT astrocytes exhibited a peak increase in soma volume of $19 \pm 1.2\%$ after 5 min, which was significantly higher than the $3 \pm 0.8\%$ increase observed in Aqp4$^{-/-}$ astrocytes ($P < 0.001$, two-tailed Student t test) (Fig. 1E). In both genotypes, the initial swelling was followed by a nearly linear volume reduction, reflecting regulatory volume decrease.

To explore whether volume recovery was dependent on the magnitude of osmotic stress, we exposed WT and Aqp4$^{-/-}$ astrocytes to artificial cerebrospinal fluid (aCSF) with 30% reduction in osmolarity. Under this condition, astrocytes from both genotypes showed continuous increase in soma volume, and magnitude of swelling was similar (Fig. 1E).

Hypoosmotic Stress Enhances Ca^{2+} Signals in Cortical Layer 1 Astrocytes in Vivo.
In vivo two-photon imaging of Glt-1–EGFP BAC transgenic mice confirmed that the Ca^{2+} indicator Rhod2 AM was taken up by astrocytes (Fig. 2 A and B). Increase in Rhod2 signal intensity was not associated with altered GFP signal (Fig. 2B). The ratio between Rhod2 and GFP signal intensities provided a more reliable measure of astrocytic Ca^{2+} signals than the Rhod2 signal (Fig. 2C), reflecting that the former measure is less sensitive to inadvertent small shifts in focal plane. In neither WT nor Aqp4$^{-/-}$ mice did frequency of astrocytic Ca^{2+} spikes change over time in the control state (0.092 ± 0.038 vs. 0.197 ± 0.065 for the first and last 15 min in WT, $n = 76$
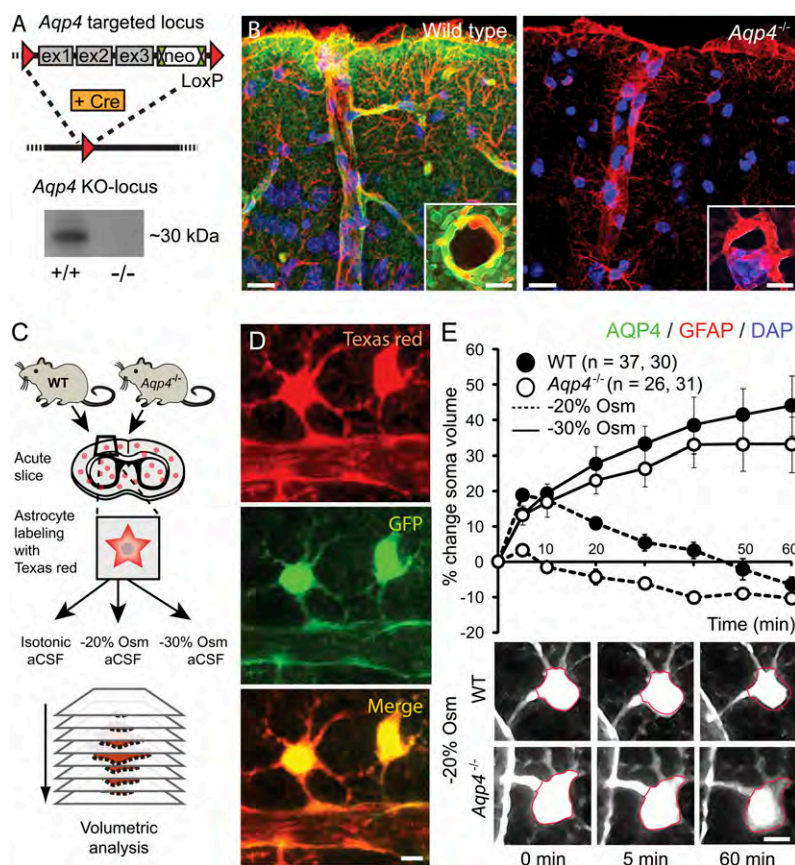
Fig. 1. *Aqp4* KO strategy and validation. (*A*) The targeted allele contained a flippase recognition target (FRT)-neomycin-FRT cassette after exon 3 and LoxP sites upstream and downstream of exons 1–3. Floxed mice were bred with Cre-expressing mice to produce mice with the *Aqp4* KO allele. Western blot confirmed the absence of AQP4 in *Aqp4⁻/⁻* mice. (*B*) Immunofluorescence micrographs of mouse cortex probed with primary antibodies against AQP4 (green) and GFAP (red) with DAPI-labeled nuclei (blue) for orientation. The AQP4 immunofluorescence signal is absent in *Aqp4⁻/⁻* mice. *Insets* display perivascular AQP4 and GFAP labeling at higher magnification. (Scale bar: 25 μm; *Inset*, 5 μm.) (*C*) Experimental design for validating the effect of *Aqp4* deletion on osmotically induced astrocyte swelling. Acute brain slices were prepared from WT and *Aqp4⁻/⁻* mouse pups. Slices were loaded with Texas red hydrazide and perfused with aCSF with normal (isotonic) or reduced osmolarity (−20% or −30% Osm). Sectional images were acquired for 3D volume analysis. (*D*) Two-photon imaging of Texas red hydrazide-loaded slices obtained from mice expressing GFP in astrocytes (*Glt-1*–EGFP BAC transgenic mice) confirmed that the dye was selectively taken up by astrocytes. (Scale bar: 5 μm.) (*E*) Exposure of acute cortical slices to 20% reduction in osmolarity (−20% Osm) induced more prominent swelling of astrocytic somata in WT (*n* = 37) than in *Aqp4⁻/⁻* mice (*n* = 26; *P* < 0.001 at 5 min, two-tailed Student *t* test). The initial swelling was followed by shrinkage reflecting regulatory volume decrease. More severe osmotic stress (−30% Osm) induced continuous swelling in both genotypes (*n* = 30 and 31). Error bars represent SEM. *Lower* shows representative images of astrocytes exposed to −20% Osm. The red ring marks the astrocyte soma circumference at baseline. (Scale bar: 5 μm.)

cells, *P* = 0.15; 0.179 ± 0.111 vs. 0.232 ± 0.076 in *Aqp4⁻/⁻*, *n* = 56, *P* = 0.55; mixed model analyses) (*Methods*).

Brain edema induced by i.p. water injection was associated with enhanced Ca²⁺ signals in WT mice (Fig. 2 *D–G*). Frequency of astrocytic Ca²⁺ spikes and percentage of astrocytes with Ca²⁺ spikes (active cells) increased already in the first 15-min period post water injection and became even higher as brain swelling progressed (Fig. 2*G*). Spike amplitude and proportion of long-lasting (≥30 s) spikes also became higher in the late phase of osmotic brain edema (Fig. 2 *E* and *F*).

Osmotically Induced Astrocytic Ca²⁺ Spikes in Vivo Are Dependent on AQP4. In the control state, frequency of astrocytic Ca²⁺ spikes did not differ between WT and *Aqp4⁻/⁻* mice (*P* = 0.69) (Fig. 2*G*). In contrast to WT, *Aqp4⁻/⁻* mice did not respond to water injection with altered astrocytic spike frequency (Fig. 2*G*). Only the proportion of active astrocytes increased somewhat in the late phase of osmotic brain swelling (Fig. 2*G*). At this stage, proportion of active astrocytes (*P* = 0.0042) and spike frequency (*P* = 0.0038) differed between WT and *Aqp4⁻/⁻* mice. In the first 15 min after water injection, these values did not differ signifi-

cantly between genotypes (*P* = 0.098 and *P* = 0.159, respectively; mixed model analyses) (*Methods*), probably reflecting the time it takes for brain swelling to develop after i.p. water injection.

Mild Hypoosmotic Stress Induces AQP4-Dependent Astrocytic Ca²⁺ Responses in Acute Cortical Slices. Supporting our in vivo findings, we found that exposing acute cortical slices to mild hypoosmotic aCSF (20% reduction in osmolarity) robustly triggered astrocytic Ca²⁺ spikes in WT mice (Fig. 3 *A* and *B*). The proportion of astrocytes that responded with Ca²⁺ spikes was much lower in *Aqp4⁻/⁻* than in WT mice (Fig. 3 *A* and *B*). Moreover, Ca²⁺ spikes in *Aqp4⁻/⁻* mice had a lower amplitude (Fig. 3*B*) and delayed onset (272 ± 9 s in *Aqp4⁻/⁻* vs. 162 ± 4 s in WT, *P* < 0.001, two-tailed Student *t* test). More severe osmotic stress (30% reduction in osmolarity) diminished the difference in responder rate between WT and *Aqp4⁻/⁻* mice (Fig. 3*B*), possibly reflecting robust astrocyte swelling in both genotypes during this condition (compare with Fig. 1*E*). However, at 30% reduction in osmolarity, the spike amplitude was still lower in *Aqp4⁻/⁻* than in WT mice (Fig. 3*B*).

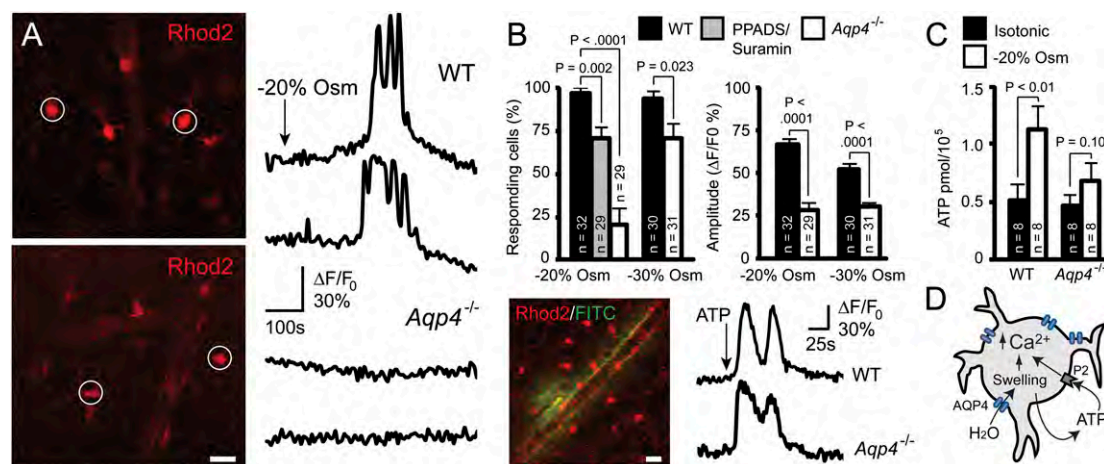**Fig. 2.** In vivo two-photon imaging of astroglial Ca^{2+} signals during hypoosmotic stress. (A) Diagram of experimental setup. Astrocytic Ca^{2+} transients were detected in anesthetized *Glt-1*–EGFP BAC transgenic mice after loading of Rhod2 AM onto the cortical surface. (B) Images show Rhod2 fluorescence in a GFP-expressing astrocyte. Because increase in Rhod2 signals were not associated with changes in GFP fluorescence, the ratio between the two signals provided a measure of the astrocytic Ca^{2+} signal. (C) During brain swelling, the ratio between Rhod2 and GFP fluorescent signal intensities (lower trace) was less sensitive to inadvertent small shifts in focal plane than Rhod2 signals (upper trace). Thus, Rhod2/GFP signals were used for reliably defining Ca^{2+} spikes [i.e., transients exceeding 20% (dashed line) of baseline]. (D) Pooled Ca^{2+} traces (measured as relative changes in Rhod2/GFP) for all astrocytes ($n = 24$) within an image field in a WT mouse subjected to i.p. water injection (indicated by arrow; 200 mL/kg) to induce osmotic brain swelling. Note increase in spike frequency and amplitude as brain edema develops. (E) In WT mice, relative spike amplitude and proportion of spikes lasting ≥ 30 s were higher in the last 15 min than in the first 15 min after water injection. (F) Traces of relative Ca^{2+} changes in WT astrocytes 30 min after i.p. water injection. The position of the respective astrocytes (confluent Rhod2 and GFP signals in yellow) is indicated in *Left*. FITC-dextran (green) was injected i.v. at the beginning of the experiment to outline the vasculature, and it confirmed vascular perfusion. Time-lapse sequence of the Ca^{2+} responses is shown in *Right*. Note the intense and long-lasting Ca^{2+} surge in the astrocytic soma and endfoot surrounding a vein. (Scale bar: 25 μm.) (G) In WT mice, the frequency of astrocytic Ca^{2+} spikes was higher already in the first 15 min after water injection compared with the control state, and it was even higher in the last 15 min of observation. In $Aqp4^{-/-}$ mice, water injection did not increase spike frequency. The percentage of astrocytes with more than or equal to one Ca^{2+} spike(s) per 15-min observation (active astrocytes) increased profoundly in WT mice after water injection. In $Aqp4^{-/-}$ mice, the number of active astrocytes increased only at the late phase of osmotic brain swelling. Error bars represent SEM. Mixed model analyses were performed using a binomial (Bernoulli) model with logit link for binary observations (passive or active cells), a Poisson model for count data (spike frequency), and a linear model for spike amplitudes (24).

AQP4-Dependent Ca^{2+} Signals Are Mediated in Part by Autocrine Purinergic Signaling. Incubating WT slices with the nonselective P2 antagonists suramin and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) delayed onset of Ca^{2+} responses to mild hypoosmotic stress (327 ± 8 s vs. 241 ± 9 s without antagonists, $P < 0.001$, two-tailed Student t test) and reduced percentage of responding astrocytes (Fig. 3B). Microinjection of ATP in cortical slices induced astrocytic Ca^{2+} responses with similar kinetics in WT and $Aqp4^{-/-}$ mice (Fig. 3B Lower) (mean amplitude = $68 \pm 2\%$ vs. $69 \pm 3\%$; time to response = 11 ± 1.2 s vs. 12 ± 1.2 s, respectively), suggesting that $Aqp4$ deletion did not interfere with signaling mechanisms downstream of purinergic receptor activation.

***Aqp4* Deletion Abrogates Osmotically Induced ATP Release from Cultured Astrocytes.** Cultured WT astrocytes exposed to hypoosmotic medium (−20% Osm) for 15 min released more ATP than those kept in isotonic solution (Fig. 3C). In contrast, cultured $Aqp4^{-/-}$ astrocytes subjected to similar stress showed no significant change in ATP release (Fig. 3C). Taken together, our data are compatible with a role for AQP4 in amplifying signaling events triggered by cell swelling (Fig. 3D).

Discussion

Brain edema formation is commonly regarded as a passive process by which water accumulates in the brain because of changes in osmotic driving forces or perturbations of the blood–brain barrier. Hence, the symptomatology and treatment of this serious condition is usually discussed in the context of the accompanying intracranial pressure changes that may eventually cause herniation and compromise blood flow to the brain. The possibility that brain edema formation sets in motion pathophysiologically relevant signaling processes has largely been overlooked. This is remarkable given the fact that cell swelling—the hallmark of cytotoxic edema—is known to activate a number of signaling cascades that may have profound effects on cell function (14). Specifically, in vitro studies have shown that swelling causes astrocytes to release neuroactive substances such as glutamate and ATP (6, 15).

This study shows that incipient edema is associated with astrocytic Ca^{2+} signals in vivo. These signals are causally linked to water influx and cell swelling, because they were significantly reduced in animals deficient in AQP4. Previously, deletion of $Aqp4$ has been shown to abrogate osmotically induced astrocytic swelling and counteract build-up of brain edema (8, 16).

Fig. 3. Osmotically induced astrocytic Ca²⁺ responses and ATP release in vitro. (*A*) Two-photon images of Rhod2 AM-loaded acute cortical slices obtained from WT (*Upper*) and *Aqp4*⁻/⁻ (*Lower*) mice. Traces shown are from astrocytes marked in *Left*. Exposure of slices from WT mice to aCSF with 20% reduction in osmolarity (−20% Osm) induced brisk astrocytic Ca²⁺ spikes. In contrast, this osmotic stress failed to elicit Ca²⁺ spikes in most AQP4-deficient astrocytes. (*B*) Quantitative analysis of astrocytic Ca²⁺ responses to osmotic stress. Deletion of *Aqp4* or blocking P2 purinergic receptors with PPADS/suramin significantly reduced the number of astrocytes that responded with Ca²⁺ spikes during exposure to −20% Osm. When more severe hypoosmotic stress (−30% Osm) was applied, a larger fraction of the *Aqp4*⁻/⁻ astrocytes responded. The amplitude of the Ca²⁺ spikes differed between the genotypes for both types of stress. Image shows intense Ca²⁺ signals in Rhod2-loaded astrocytes after microinjection of ATP and FITC-dextran (green; to verify injection) into the slice. Representative traces from WT and *Aqp4*⁻/⁻ mice are shown. (*C*) Cultured astrocytes exposed to hypoosmotic media (−20% Osm, 15 min) released significantly more ATP than those kept in isotonic media. Astrocytes from *Aqp4*⁻/⁻ mice did not show osmotically induced ATP release. *P* values were obtained by two-tailed Student *t* test. Error bars represent SEM. (Scale bar: 25 μm.) (*D*) Diagram showing proposed involvement of AQP4 in astrocyte signaling cascades during hypoosmotic stress. AQP4-mediated water influx triggers Ca²⁺ transients, partly by promoting release of ATP and activation of P2 purinergic receptors.

The in vivo analyses were complemented with monitoring of blood flow in the microvascular bed. Despite absence of overt changes, it is difficult to rule out small alterations in cerebral perfusion caused by the incipient brain edema. Thus, it was deemed necessary to include complementary in vitro studies in slices. Such studies also allowed us to dissect the mechanisms underlying the AQP4-sensitive Ca²⁺ responses. Analyses in acute cortical slices supported the data obtained in vivo. Notably, slices exposed to hypoosmotic media displayed Ca²⁺ signals in astrocytes reminiscent of those seen in vivo. These signals were attenuated after *Aqp4* deletion. The attenuation was particularly pronounced at 20% decrease in osmolarity.

Previous in vitro studies have shown that activation of purinergic receptors triggers astrocytic Ca²⁺ transients (17–19). We hypothesized that Ca²⁺ signals elicited during edema formation depend—at least in part—on ATP release from swollen astrocytes. Application of P2 antagonists to acute cortical slices supported this view. The quantitative analysis indicated that, in ∼25% of WT astrocytes, the Ca²⁺ response was contingent on ATP signaling. Obviously, additional mechanisms are at play and contribute to the observed Ca²⁺ signals. Stretch-sensitive receptors are likely to be among these mechanisms.

Next, we set out to resolve whether astrocytes could serve as a source of ATP. In cultured astrocytes, osmotic stress induced ATP release, and this release was abolished after *Aqp4* deletion. Taken together, the data suggest that AQP4 not only mediates water influx but also is essential for initiating signaling events associated with edema formation. This may explain the rather pronounced protective effect of *Aqp4* KO or AQP4 mislocalization in stroke models (8, 20). It is well-known that water passes through the lipid bilayer of the plasma membrane (although to a limited extent compared with the water flux through aquaporin channels) and that diffusion also occurs through the thin slits that separate the astrocyte endfeet. In AQP4-deficient mice, a discrepancy between the extent of water transport restriction on the one hand and the protective effect in stroke on the other hand can easily be explained if loss of AQP4 also interferes with signaling mechanisms that exacerbate the pathological outcome.

Edema formation and cytotoxicity likely engage in a vicious cycle, where cell swelling causes release of cytotoxic compounds that, in turn, lead to tissue damage and more swelling. ATP is known to act as a cytotoxic compound in stroke, as judged by a number of in vitro and in vivo studies (21). Thus, an early-stage intervention with AQP4 inhibitors would interfere with this vicious cycle by counteracting not only the swelling per se but also deleterious secondary events like ATP release. Further studies are required to resolve whether AQP4 is also involved in swelling-activated glutamate efflux through volume-sensitive channels (6).

An obvious question is whether the effect of *Aqp4* deletion solely depends on the change in swelling response or whether AQP4 (alone or in combination with other molecules) serves as an osmosensor upstream of the above signaling events. To distinguish between these possibilities, we exposed acute slices to an osmotic stress (30% reduction in osmolarity) severe enough to override the mechanisms that normally limit transmembrane water transport, as evidenced by the reduced sensitivity to *Aqp4* deletion. With an osmotic stress at this scale, the percentage of astrocytes that responded with Ca²⁺ spikes was nearly as high in the *Aqp4*⁻/⁻ animals as in WT. This observation is consistent with the idea that the Ca²⁺ signals are elicited by the AQP4-induced swelling response rather than through an osmoreceptor response.

Conclusion

Our study has revealed that induction of brain edema sets in motion specific signaling events in brain cells. Notably, we have shown by in vivo two-photon imaging that osmotic stress and edema formation are associated with brisk Ca²⁺ signals in cortical astrocytes. This observation prompted us to resolve whether these signals are dependent on AQP4, which is assumed to constitute the main influx route for water at the brain–blood interface. Using a *Aqp4*⁻/⁻ line, we show that deletion of *Aqp4* interferes with the frequency and amplitude as well as the duration of the Ca²⁺ signals observed. Taken together with complementary analyses in reduced experimental models, our data are consistent with the idea that AQP4-mediated cell swelling is inextricably coupled with activation of signaling pathways that

may profoundly affect the pathological and pathophysiological outcome in clinical conditions associated with brain edema.

Methods

Mice. $Aqp4^{-/-}$ mice were generated by GenOway by cloning and sequencing of a targeted region of the murine $Aqp4$ gene in a 129/Sv genetic background. The strategy was to design a targeted locus allowing us to delete exons 1–3 to avoid any expression of putative splice variants. Hence, a flippase recognition target (FRT)-neomycin-FRT-LoxP–validated cassette was inserted downstream of exon 3, and a LoxP site was inserted upstream of exon 1 as depicted in Fig. 1A. After homologous recombination in ES cells, ES-cell injection into blastocytes, and generation of chimeras, heterozygous floxed mice were obtained by breeding chimeras with C57BL/6J females. Heterozygous floxed mice were bred with C57BL/6J Cre expressing mice to generate mice heterozygous for the KO allele, $Aqp4^{+/-}$. The $Aqp4^{+/-}$ mice were then backcrossed with C57BL/6J mice for five generations before intercrossing to yield $Aqp4^{-/-}$ and $Aqp4^{+/+}$ (WTs). For acute cortical slice experiments, we also used C57BL/6J pups from Jackson Laboratory as WT controls. For in vivo experiments, we used $Aqp4^{-/-}$ and WT mice expressing EGFP in astrocytes. These mice were generated by breeding $Aqp4^{-/-}$ and WT mice with BAC promoter reporter transgenic mice that express EGFP under the control of the natural $Glt-1$ promoter (13). The latter mice were provided by J. D. Rothstein (Johns Hopkins University, Baltimore, MD).

Western Blot and Immunohistochemistry. After homogenization and solubilization, extracts of $Aqp4^{-/-}$ and WT brains were loaded onto a 10% SDS/PAGE gel and subsequently transferred onto 0.2-μm poly(vinylidene difluoride) membrane (Bio-Rad). The membrane was probed with 0.02 μg/mL goat anti-AQP4 (Cat# sc-9888; Santa Cruz Biotechnology), developed using alkaline phosphatase substrate (ECF Western blotting reagents; Amersham Pharmacia), and visualized with a Typhoon Variable Mode Imager (Amersham Pharmacia). Fixation of mice, preparation of tissue slices, and immunohistochemistry were performed as described previously (22). We used a monoclonal antibody against GFAP (G3893, 1:100; Sigma) and a polyclonal antibody against AQP4 (AB3068, 1:100; Chemicon International).

Animal Preparation for in Vivo Imaging. Eight- to twelve-wk-old WT and $Aqp4^{-/-}$ mice expressing EGFP in astrocytes were anesthetized with urethane and α-chloralose (1 g/kg and 50 mg/kg i.p., respectively), intubated, and artificially ventilated with room air using a small animal ventilator (SAR830; CWE) set to ~100 breaths/min with a tidal volume of 0.3–0.4 mL. Body temperature was kept at 37 °C by a temperature-controlled heating blanket. A craniotomy (3 mm in diameter) was made over the cortex 1 mm lateral and 0.5 mm posterior to the bregma, and the dura was removed. The Ca^{2+} indicator Rhod2 AM (2 mM; Invitrogen) was loaded to the exposed cortex for ~50 min. After washing for 10 min with aCSF, the craniotomy was covered with 1% agarose in aCSF and sealed by a coverslip. The femoral artery was cannulated for continuous monitoring of mean arterial blood pressure and analysis of blood gases. Only mice with blood gases within the physiological range (pO_2 = 80–150 mmHg, pCO_2 = 30–45 mmHg, pH 7.25–7.5) were included. To outline the vasculature, we administered FITC-dextran (2,000 kDa, ~0.4 mL, 2.5% in saline; Sigma) intravenously. WT and $Aqp4^{-/-}$ mice (n = 6 for each genotype) were injected with distilled water (200 mL/kg) i.p. immediately before imaging. Mice not receiving water injections (n = 3 for each genotype) served as controls.

In Vivo Two-Photon Imaging. A Mai Tai laser (SpectraPhysics) attached to a confocal scanning system (Fluoview 300; Olympus) and an upright microscope (IX51W; Olympus) was used for in vivo imaging, as previously described (23). A 20× (0.9 NA) water immersion lens with 3× additional zoom was used to image astrocytes in cortical layer 1. Excitation wavelength was 860 nm, and emission was collected at 575–645 nm. Dual-channel (Rhod2 and GFP) images with 512 × 512-pixel frames were acquired in a region with intact capillary perfusion every 5 s for 60 min or until capillary perfusion stopped. The low sampling rate was used to avoid photo damage. The two-photon laser power was adjusted to a power that was less than 40 mW at the sample. Two of six WT and one of six $Aqp4^{-/-}$ mice subjected to water injection died before 60 min (at 30, 48, and 55 min, respectively). During brain swelling, the focus was adjusted for persistent imaging of the same area. A Ca^{2+} spike was defined when the relative ratio between the Rhod2 and GFP signal intensities exceeded 20% of baseline over a 10-min recording period. Frequency of spikes was calculated per astrocyte per 15 min. Because of the low frequency of Ca^{2+} spikes in the control state, we averaged data for two consecutive 15-min periods. Active astrocytes were defined as cells that had

more than or equal to one Ca^{2+} spike(s) per 15 min. Values are expressed as mean ± SEM. Mixed model analyses were performed using a binomial (Bernoulli) model with logit link for binary observations (passive or active cells), a Poisson model for count data (spike frequency), and a linear model for spike amplitudes (24). A hierarchical structure was assumed for all these models, with random effects representing variation between animals and cells inside animals. All experiments were approved by the Institution Animal Care and Use Committee of University of Rochester.

Preparation of Acute Cortical Slices and Dye Loading. Coronal cortical slices were prepared from 10- to 20-d-old mice of either sex as described previously (22, 25). In brief, the brains were submerged in gassed (95% O_2 and 5% CO_2) ice-cold cutting solution, and coronal slices (400 μm) were cut on a Vibratome (TPI). Slices were incubated for 20 min in aCSF and then loaded with either Rhod2 AM (2 mM) or Texas red hydrazide (1.5 μM) in aCSF at 35 °C for 50 min. The aCSF, gassed as described above, contained (in mM) 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 26 $NaHCO_3$ (pH 7.4).

Two-Photon Imaging in Acute Cortical Slices. Dye-loaded slices were transferred to a recording chamber (1.5 mL), held in place by a nylon-threaded metal grid, and continuously perfused with aCSF (room temperature) at a rate of 2 mL/min. A multiphoton laser scanning microscope as described above was used for imaging (excitation wavelength = 820–860 nm; emission collected at 575–645 nm). A 60× (0.9 NA) water immersion lens with 6–10× additional zoom was used for the volume assessment, whereas a 20× (0.9 NA) water immersion lens was used for calcium imaging. All experiments were performed at room temperature. After imaging in normal aCSF, we switched the perfusion solution to hypoosmotic aCSF. This solution differed from the control aCSF only with respect to the NaCl concentration, which was either reduced by 20% or 30% (NaCl = 100.8 or 88.2 mM, respectively). Osmolarities were verified by freezing-point depression.

Volumetry. Astrocytes with endfeet visibly extending to vessels at a tissue depth >40 μm were selected, and 3D image stacks were collected; frame sizes of 256 × 256 at intervals of 1.5–3.0 μm in the z direction were collected with an acquisition time of <20 s using minimal laser power (<40 mW). Images were acquired for a 60-min period and then analyzed for changes in soma volume using custom-made software (Matlab Inc.). A median filter with a radius of 5 pixels was used to reduce background noise. A region of interest was defined around the soma in maximum intensity projections using fixed landmarks for all time points within an experiment. Pixels over a certain threshold within this region were counted for each xy frame in the z stack. Laser power, photomultiplier tube sensitivity, and thresholds were kept constant for each image sequence. Automated thresholding was performed by normalizing pixel intensity to a decay constant extracted from an intensity histogram of each image. The sum of pixels for each xyz stack was then compared with the sum at baseline. Values are expressed as mean ± SEM. Intragroup and intergroup analyses were performed using a two-tailed Student t test.

Calcium imaging. Time-lapse images of astrocytic Ca^{2+} signals were recorded in the slices every 5 s. Images were acquired 1 min before the solution change as a baseline and 10 min after; 512 × 512 frames were acquired every 5 s using minimal laser power (<40 mW). Changes in Rhod2 intensity ($\Delta F/F_0$) were analyzed using custom-made software (Matlab Inc.) within a manually defined region of interest (10-μm-diameter circle for soma). Relative Rhod2 increases >20% (or >2 SDs) above baseline value were defined as a Ca^{2+} spike. After administration of ATP, the Ca^{2+} response was imaged for 168 s, one frame per 1.12 s, using 512 × 512 frames. Values are expressed as mean ± SEM. Intragroup and intergroup analyses were performed using a two-tailed Student t test.

Drug delivery. For experiments with P2 purinergic receptor antagonists, the slices were exposed to suramin (100 μM; Tocris Bioscience) and PPADS (30 μM; Tocris Bioscience) >30 min before imaging and hypoosmotic challenge. Slices exposed to microinjected ATP were perfused with control aCSF. A fine electrode filled with aCSF containing 500 μm ATP was inserted 40–80 μm into the slice. After a 33.6-s baseline recording, ATP was puffed through the electrode using a Picospritzer (10 psi, 100 ms; Parker Instrumentation); 1% FITC was used to visualize the puff.

ATP Release from Cultured Astrocytes. Serum-free media (control) or solution with 20% reduction in osmolarity (by removing NaCl) was added (400 μL; 2×) to astrocyte cultures derived from WT or $Aqp4^{-/-}$ mice. After 15-min exposure, 350-μL samples were collected (450 μL remained in the wells). ATP content in samples collected from cultures grown in 24-well tissue culture plates was measured by using a bioluminescent ATP assay kit (Sigma) and a Victor2 plate reader (Wallac) and was normalized to the cell number (26). A total of eight plates were examined for each genotype and their re-

spective control. Values are expressed as mean $\pm$ SEM. Intergroup analyses were done using a two-tailed Student t test.

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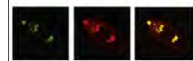
Review

Brain connexins in demyelinating diseases: Therapeutic potential of glial targets

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A R T I C L E   I N F O

A B S T R A C T

Several demyelinating syndromes have been linked to mutations in glial gap junction proteins, the connexins. Although mutations in connexins of the myelinating cells, Schwann cells and oligodendrocytes, were initially described, recent data have shown that astrocytes also play a major role in the demyelination process. Alterations in astrocytic proteins directly affect the oligodendrocytes' ability to maintain myelin structure, and associated astrocytic proteins that regulate water and ionic fluxes, including aquaporins, can also regulate myelin integrity. Here, we will review the main evidence from human disorders and transgenic mouse models that implicate glial gap junction proteins in demyelinating diseases and the therapeutic potential of some of these targets.

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1.   Introduction

1.1.   Composition and function of myelin in the nervous system

Myelination is essential for brain function in mammals, as it speeds up transmission of neural information. Several sheaths of myelin surround every single axon. This creates an insulating layer of fat with regular discontinuities called nodes of Ranvier. These nodes concentrate the necessary machinery to propagate action potentials and allow the electrical signals to travel in a saltatory manner to reach other cells located hundred of mms away within milliseconds (Sherman and Brophy, 2005).

Although, in principle, the concept of layers of lipid membranes for insulation sounds simple, myelin formation and organization is a rather complex process. Apart from the unique lipid composition of its plasma membrane, several proteins exclusive to myelin serve as structural support within the myelin membranes. Proteolipid protein (PLP) and myelin-associated glyocoprotein (MAG) are some of the main integral proteins in myelin (Nave, 2010) although their exact role is still elusive. In the intracellular space, myelin basic protein (MBP), one of the most critical myelin proteins, creates a framework for attachment, not only of lipids but also of diverse membrane proteins including ionic channels, transporters, gap junctions as well as cytoskeletal proteins, and signaling molecules.

The complexity of white matter organization suggests that myelin contributes not only to insulation but also to signaling within the myelinating cell and axon. For example, the intimate neuro-glial interaction obtained through myelination has proven crucial for axonal integrity and survival. In addition, myelination also allows energy savings by concentrating critical ionic channels in a very restricted area of the axons, thereby reducing the amount of ATP consumed in restoring ionic gradients after every action potential (Nave, 2010).

Many different human disorders have been described to date that affect either the production or the maintenance of myelin. Some of these demyelination pathologies have been linked to a particular group of proteins – the connexins (Cxs) – that form intercellular gap junction channels with adjacent cells, connecting their cytoplasms. These channels allow the exchange of ions and small metabolites up to 1 kDa in size and contribute to cooperative metabolism among cells, electrical coupling and spatial buffering (Bruzzone et al., 1996). Alterations in connexins present in the myelinating glial cells (forming intercellular junctions in oligodendrocytes and autaptic – within themselves – in Schwann cells) all promote demyelination diseases. Interestingly, connexins present in the astrocytes, the major macroglial cell type in the nervous system and not traditionally associated with the myelination process, also contribute to some myelin pathologies.

Here, we will discuss the evidence that supports a role for connexins and related proteins present in both oligodendrocytes and astrocytes in myelin disorders. We will also discuss putative signaling mechanisms that could be involved and the potential for therapeutic intervention based on these targets.

2.   Oligodendrocyte-mediated demyelination: connexins

Oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) are the cells involved in synthesizing, organizing and wrapping myelin around the nerves. One single oligodendrocyte can wrap many axons, giving a web-like appearance to these cells in the white matter (Nave, 2010). Oligodendrocytes and Schwann cells express three different connexins: Cx47, Cx32 and Cx29, although only the first two are believed to form gap junction channels (Ahn et al., 2008). Whereas, Cx47 forms extensive gap junctions with astrocytes in soma and outer myelinated fibers, Cx32 is the most abundant within the layers of myelin itself ("reflexive" or "autologous" gap junctions), between loops of the myelin sheath in individual oligodendrocytes and Schwann cells (Kamasawa et al., 2005),
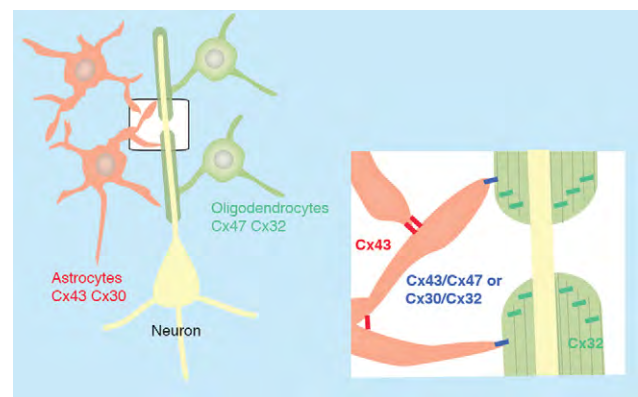

Fig. 1 – Schematic of the connexins involved in formation of astrocytic, oligodendrocytic and astro-oligodendrocytic gap junctions. Astrocyte–astrocyte gap junctions are composed primarily of Cx43 (red), with a minor contribution of Cx30. Oligodendrocyte–oligodendrocyte gap junctions are primarily assembled by Cx32 (green), whereas gap junctions connecting astrocytes and oligodendrocytes are composed of a mixture of Cx43/Cx47 or Cx30/Cx32. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

although it can also form gap junctions with other astrocytic connexins (Fig. 1). These more direct pathways between the myelin layers allow a much shorter route for metabolite exchange.

Several human disorders are caused by defects in oligodendrocyte connexins. Below we discuss some of the information we have learnt from the study of human diseases as well as transgenic mice models.

2.1. Charcot–Marie tooth disease (CMT1X)/ X-linked progressive peripheral neuropathy – Cx32 in Schwann cells (PNS)

Mutations in the gene that encodes Cx32 cause X-linked Charcot–Marie-Tooth disease, a peripheral neuropathy characterized by the loss of myelinated fibers accompanied by axonal alterations, axon regeneration and altered myelin thickness and loosening (reviewed in Kleopa 2011). Although CMT1X is primarily a peripheral neuropathy that starts in distal limbs, there are also some CNS manifestations that may include cerebellar ataxia or encephalopathy under stress conditions (Kleopa 2011).

2.2. Demyelination in leukodystrophies – Pelizaeus–Merzbacher-like disease or milder spastic paraplegia – Cx47 and Sox10

The term leukodystrorephy refers to a group of disorders that result from abnormalities in myelin, with failure to form at all as in Pelizaeus–Merzbacher disease (PMD) or to maintain it as in Krabbe disease (Duncan et al., 2011).

In most cases, mutations in oligodendrocyte proteins are causative of the disease (proteolipid protein – PLP – in PMD; the lysosomal enzyme galactocerebrosidase – GALC gene- in Krabbe disease, to cite a few). In 20–50% of the cases of PMD, however, no recognized mutation in the PLP gene is found.

In Pelizaeus–Merzbacher-like disease (PMLD), mutations in the gap junction protein Cx47 are the main abnormality identified to cause severe CNS myelin deficiency (Uhlenberg et al., 2004). Patients with PMLD often exhibit nystagmus, impaired motor development, ataxia and progressive spasticity and sometimes, mild peripheral neuropathy.

Mutations in the Cx gene produce much more severe phenotypes than eliminating the entire Cx47 gene altogether in mice (see below). An explanation for this could be the redundant nature of the connexin family members, as oligodendrocytes also express two other connexins that can compensate for the absence of Cx47. In the case that the connexin is present but not fully functional, this compensatory mechanism is halted. Deficiencies may then arise due to improper trafficking of the mutant connexin proteins to the membrane and/or faster degradation.

Recently, a new mutation affecting the Cx47 promoter has been described in a patient with mild PMLD. This mutation affects the binding site of the high mobility group transcription factor SOX10 (Osaka et al., 2010), and SOX10 mutations also cause hypomyelination (Pingault et al., 1998). These facts strengthen the notion that proper transcription of the Cx47 gene is critical for CNS myelination.

2.3. Demyelination in mouse models with mutant oligodendrocyte connexins – Cx32 and Cx47

To help decipher the underlying mechanism by which connexins influence myelination and further explore the involvement of connexins in demyelination, several mouse models have been created that can replicate the human pathologies.

Mice lacking Cx32 or Cx47 are viable, and no obvious demyelination is observed (Nelles et al., 1996; Odermatt et al., 2003; Menichela et al., 2003) except for some myelin vacuolation. However, Cx32/Cx47 double deficient mice exhibit much more severe nerve fiber vacuolation, loss of myelin sheaths, oligodendrocyte cell death and ultimately death before two months of age (Odermatt et al., 2003; Menichela et al., 2003).

Similarly, mice transgenic for some disease-linked human Cx32 mutations show demyelinated peripheral neuropathy as the one observed in CMT1X (Sargiannidou et al., 2009). Interestingly, the localization of the two other connexins expressed in oligodendrocytes or Schwann cells, Cx47 or Cx29, is not affected in any of these mice, suggesting that the loss of one single connexin in myelin may be partly compensated by an increase in another one (Li et al., 2008).

3. Astrocyte-mediated demyelination: connexins, AQP4 and potassium

Although demyelination appears more related to defects in the myelinating cells and the myelin structure itself, several recent reports suggest a critical role for astrocytes in certain demyelinating diseases, highlighting the importance of these cells in all cellular brain interactions. Astrocytes are the most abundant cell type in the nervous system, and provide metabolic and structural support to neurons, regulate potassium and water homeostasis, glucose uptake and are also implicated in modulating certain aspects of neuronal function (Allaman et al., 2011; Nedergaard and Verkhratsky, 2012). Fibrous astrocytes, the astrocytes present in white matter, are unique because they contain higher GFAP expression, have fewer and thinner processes and do not organize in domains like the protoplasmic astrocytes in gray matter (Oberheim et al., 2009), suggesting a more structural rather than functional role. We will next review the main evidence that implicate astrocytic gap junction proteins in demyelinating diseases (Table 1).

3.1. Demyelination in leukodystrophies – Alexander's disease and vanishing white matter – GFAP and other unknown proteins

A very particular picture emerges from another leukodystrophy: Alexander's disease, because it is the only known human disorder caused by mutations in an astrocytic protein, glial fibrillary acidic protein (GFAP), the main intermediate filament protein in mature astrocytes. In early onset type I Alexander's disease, extensive cerebral white matter abnormalities occur, with frontal predominance of periventricular and perivascular lesions. Type 2 Alexander's disease has, primarily, an adult onset and affects mostly the bulbospinal system (Sawaichi, 2009).

Table 1 – Description of the main demyelinated disorders related to connexin/astrocytic proteins.

Disease	Protein involved	Reference in text
Oligodendrocyte/Schwann cells proteins		
Charcot–Marietooth disease (CMT1X)	Cx32	Kleopa (2011)
Pelizaeus–Merbacher-like disease (PMLD)	Cx47, SOX10	Uhlenberg et al. (2004); Osaka et al. (2010)
Astrocytic proteins or proteins that affect astrocytes		
Alexander's disease	GFAP	Brenner et al. (2009); Sawaichi (2009)
Vanishing white matter syndrome (VWM)	eIf2B	Bugiani et al. (2010)
Osmotic demyelination syndrome	Cx43 and Cx47	Gankam et al. (2011)
Neuromyelitis optica	AQP4	Lennon et al. (2004)
Balo's disease and multiple sclerosis	AQP4	Kira (2011)
Megalencephalic leukoencephalopathy with subcortical cysts (MLC)	MLC	Leegwater et al. (2001)
	GlialCAM	LópezHernández et al. (2011)

At the molecular and cellular levels, Alexander's disease is characterized by massive protein accumulations called Rosenthal fibers in astrocytes, large aggregates of GFAP mutant protein accompanied by two heat shock proteins HSP27 and αB-crystallin. These Rosenthal fibers are often abundant in fibrous astrocytes of subcortical white matter. Other effects on astrocytes include reduced levels of glutamate transporter GLT1 and increased lipid peroxidation and iron accumulation, indicative of oxidative stress. Although mutant astrocytes are the main cause of the disease in 90% of the cases and become very reactive, there is loss of axons and myelin in variable regions. The very highly reactive astrocytes are, paradoxically, spared of cell death (Brenner et al., 2009).

Similar to Alexander's disease, hypomyelination/vanishing white matter syndrome (VWM) and Canavan disease are also linked to defective astrocytes but much more indirectly. In VWM, one of the leukodystrophies most prevalent in childhood, mutations in the eukaryotic translation initiation factor 2B (eIf2B) have been identified as the cause of the disease. Despite the ubiquitous function of this housekeeping protein on controlling protein synthesis, it is surprising that VWM patients exhibit mostly a neuropathy characterized by the presence of diffuse, spongiform myelin, which is dramatically diminished (Bugiani et al., 2010). Most intriguingly, oligodendrocytes are increased in number (van Haren et al., 2004), whereas astrocytes are decreased and the few left present an atypical appearance (Dietrich et al., 2005). Thus, this gliopathy has been proposed to combine severe myelin deficiency with an inability of astrocytic gliosis to contain the extended damage (Bugiani et al., 2010).

The mechanisms by which altered astrocytes in these conditions promote white matter changes and affect oligodendrocytes and axons are presently unknown, although it is tempting to speculate that connexin proteins might be important mediators of these effects.
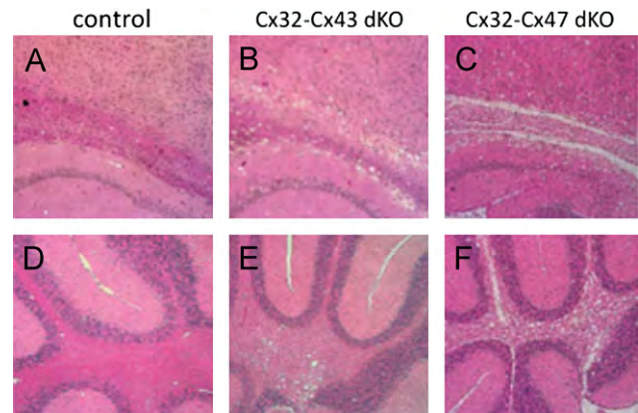

Fig. 2 – Histophatology (H&E staining) of gray and white matter of mice brain lacking the astrocyte and oligodendrocyte connexins. Low power views of sagittal sections of control mice (A, D), dKO of Cx32–Cx43 (B, E) or dKO of Cx32-Cx47 (C, F). Double mutants for astrocyte and oligodendrocyte connexins show myelin vacuolation in the corpus callosum (B, C) and cerebellar white matter (E, F) compared to controls (A, D).

In the next sections we will review some of the evidence that implicate these proteins in astrocyte-mediated demyelination.

3.2. Demyelination mediated by astrocytic gap junction proteins in mouse models – Cx43 and Cx30

Astrocytes establish abundant gap junctions among themselves via two main connexins, Cx43 and Cx30 (Nagy et al., 2001). Given that oligodendrocyte connexins are not expressed in astrocytes and that astrocytes are barely coupled among themselves in white matter of young mice (Maglione et al., 2010), it is somewhat surprising that astrocytic connexins are involved in demyelinating diseases. Although as many as 77% of the cells displaying gap junctional coupling in white matter form among oligodendrocytes (via Cx47) or within themselves (via Cx32), heterotypic coupling has also been detected between oligodendrocytes and astrocytes, albeit in lesser amounts (Maglione et al., 2010). Some evidence indicates that these heterotypic gap junctions form via Cx43/Cx47 on the one hand and Cx30/Cx32 on the other (Li et al., 2008; Maglione et al., 2010).

Still, these astro-oligodendrocyte gap junctions have enough significance to strongly affect myelination. Mutations in Cx43 or Cx30 alone does not show signs of abnormal myelination (Nakase et al., 2004; Dere et al., 2003). However, double mutants for Cx43/Cx30 show glial "edema" and myelin vacuolation in white mater, with the hippocampus CA1 region the only area where obvious pathology is found in gray matter (Lutz et al., 2009). Some sensorimotor and spatial memory deficits are also observed in these double knockout mice with no effect on life span. To address the true impact of the astrocytic connexins, Magnotti et al. (2011) developed mice mutant for one oligodendrocyte connexin and one astrocytic connexin: dkoCx43/Cx47 or dkoCx43/Cx32 (Fig. 2). Surprisingly, only the Cx43/Cx32 dKO exhibited myelin vacuolation accompanied by little effect on oligodendrocytes but marked increased in astrocytic cell death. This translated

into seizure activity and early mortality. Thus, astrocytic connexins may indirectly impact myelin integrity by compromising astrocytic survival.

3.3. Osmotic demyelination syndrome – Cx43 and Cx47

Osmotic demyelination syndrome (ODS) occurs during the process of trying to correct chronic hyponatremia. During hyponatremia (low sodium levels outside the cells), the fall in serum osmolarity driven by the fall in sodium levels causes water to move into the cells, posing the risk of cerebral edema. In an attempt to correct this imbalance, there may be too rapid osmotic changes that translate into neurological symptoms known as ODS. This syndrome is characterized by demyelinating lesions in the CNS, especially in the pons, and is preceded by massive death of astrocytes (Gankam et al., 2011). In the early events of the process (12 h after correction of hyponatremia has been initiated), there is substantial downregulation of astrocytic Cx43 on the one hand, and oligodendrocytic Cx47 on the other. None of these changes are observed in the non-corrected hyponatremic brain (Gankam et al., 2011). Changes in the blood brain barrier have been also reported.

3.4. Demyelination in neuromyelitis optica – alterations in astrocytic aquaporin-4 (AQP4)

Neuromyelitis optica (NMO) is an inflammatory autoimmune disease usually restricted to the optic nerve and spinal cord and is characterized by extensive myelin loss in both gray and white matter areas (Hinson et al., 2010). A specific autoantibody neuromyelitis optica-immunoglobulin G (NMO-IgG) that binds the water channel protein AQP4 has been identified as the primary cause of this pathology (Lennon et al., 2004). AQP4 is the main water regulator of astrocytes and is not expressed in oligodendrocytes. Thus, alterations in this astrocytic protein directly affect the capability of oligodendrocytes to maintain myelin integrity. The exact mechanism for this effect is not clear although complement activation, downregulation of AQP4 and of the glutamate transporter EAAT2 and death of oligodendroctyes all occur in this disorder (Hinson et al., 2008; Marignier et al., 2010).

3.5. Balo's disease and multiple sclerosis (MS) – AQP4 alterations

An antibody-independent selective loss of AQP4 is also observed in multiple sclerosis (MS) and Balo's disease. MS is an autoimmune disease where the body's immune system attacks the myelin sheath causing nerve damage. Balo's disease is a variant of MS in that the demyelinating tissue forms concentric layers (Kira, 2011). Similar to NMO, these two diseases also feature extensive loss of AQP4 and highly hypertrophic astrocytes. Most importantly, unpublished observations suggest that these three disorders also share the disappearance of connexins (Kira, 2011). This evidence points again to a role for astrocytic proteins in the maintenance of myelination.

Interaction of AQP and connexins was first reported in the lens (Yu and Jiang, 2004) where mutations in members of these two families of proteins (Cx50 and AQP0) independently cause human congenital cataracts (reviewed in Huang and He, 2010). Liu et al. (2011) have recently demonstrated that AQP0 has a direct role in the regulation of functional gap junction channels by influencing cell–cell adhesion. In brain astrocytes, AQP4 knockdown produced a strong downregulation of Cx43 with concomitant reduction in cell coupling (Nicchia et al., 2005). However, these effects were exclusive of mouse astrocytes with no similar alterations in human or rat cells. Thus, although it is still not clear how consistent the interaction between connexins and aquaporins is among different connexin types and different species, and the relevance of these interactions to human disorders remains uncertain, it is possible to suggest that a functional relationship between the AQP water channels and the connexins might explain the effects of AQP4 defects in demyelination.

3.6. Megalencephalic leukoencephalopathy with subcortical cysts (MLC) – MLC and GlialCAM proteins

Further evidence that supports a critical role for the astrocytic regulation of water and ion fluxes in the maintenance of myelin integrity comes from this leukodystrophy. In MLC, myelin vacuolation is accompanied by increased water content of the brain (van der Knaap et al., 1995, 1996). Mutations have been found in the MLC1 protein, a transmembrane astrocytic protein with low degree of homology to ion channels, and in the GlialCAM adhesion protein (Leegwater et al., 2001; López-Hernández et al., 2011). Recently, GlialCAM has been identified as a binding partner of the Cl- channel CIC-2 (Jeworutzki et al., 2012) and is responsible to target this channel to astrocytic cell junctions, endfeet around vessels and myelin. Interestingly, MLC1 and the actin-binding protein zonula occludens 1 (ZO-1) colocalize in humans (Duarri et al., 2011) and ZO-1 also interacts physically with some connexins (Li et al., 2004).

Further studies on the inter-dependence between connexins, aquaporins, ion channels and cell–cell junction proteins will help to determine the impact of these proteins in demyelination.

4. Could astrocytic connexins and interacting proteins be a new family of targets for therapeutic intervention in myelinating diseases?

4.1. Connexins and inflammation

An interesting aspect of NMO and related disorders is the involvement of a robust T-cell response against major myelin proteins. This, in turn, initiates a cascade of CNS inflammation, with increased levels of T-helper-1 (Th1), interferon γ (IFNγ) and of the cytokines IL-17 and IL-8 (Kira, 2011) that correlate positively with lesion size.

Sharma et al. (2010) have also shown involvement of an inflammation step in a model of lipopolysaccharide (LPS)-induced demyelination. Injection of LPS into white matter triggers initial microglial activation, followed by a strong

astrocytic reaction. This, in turn, is accompanied by the loss of AQP-4 and connexins and consequent myelin degeneration.

Inflammation can converge negatively on gap junctions in several ways. For example, reactive glia release cytokines, like tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β), that affect connexin expression and gap junction permeability in astrocytes (Meme et al., 2006). Interestingly, these same cytokines regulate oppositely hemichannel activity increasing membrane permeability (Retamal et al., 2007). In a model of spinal cord injury, O'Carroll et al. (2008) used mimetic peptides to block hemichannel activity and found that these peptides were able to reduce swelling, the number of reactive GFAP+ astrocytes, and the loss of NeuN+ neurons. Huang et al. (2012) have, in addition, shown that Cx43/Cx30 mediated ATP release is implicated in the post-traumatic inflammation after spinal cord damage. The involvement of Cx43/Cx30 in reduction of inflammation during injury does not seem to apply to all inflammatory processes as deletion of these two connexins did not impact the extent of inflammation in a mice model of experimental autoimmune encephalomyelitis (Lutz et al., 2012).

It is possible that an initial inflammatory step that alters gap junction communication precedes connexin downregulation, alterations in connexin-mediated astro-oligodendrocyte interactions and, ultimately, loss of myelin with subsequent reduction of inflammatory damage. This cascade of events may be secondary to the initial damage in an attempt to contain further inflammation and would place inflammation as an important causative aspect to keep in mind as a trigger for CNS demyelination when astrocyte dysfunction is involved. It also highlights the importance of hemichannel blocking agents as putative therapeutic agents against myelination alterations.

4.2.   Connexins and potassium channels

The functional role of astrocytic connexins in the brain has been always elusive given the lack of reagents that exclusively block gap junctional channels. New studies in mutant mice deficient for the two astrocytic connexins have allowed a better insight on how the absence of Cx30 and Cx43 affect brain structure and function. Wallraff et al. (2006) has found that astrocytic coupling allowed for a rapid removal of extracellular potassium after neuronal activity and aided its redistribution to areas of lower concentration (potassium "siphoning"). Similarly, Pannasch et al. (2011) have found that the network of Cx30/Cx43-coupled astrocytes modulate synaptic transmission in the hippocampus by removing extracellular glutamate and potassium after synaptic activity. The size of the astrocytic network, a direct correlation of gap junctional coupling, seems crucial to control synaptic strength.

Recently, the concept of "translational" channelopathies is taking shape as more and more disorders present deficits based on secondary changes in ionic channels, more specifically, sodium and potassium ionic channels. The upregulation and downregulation of ionic channels as a consequence of disease are post-translational in nature but can determine the progression of the disease. For example, levels of the sodium channel Nav1.8 are altered in MS and both Kv1.1 and Kv1.2 potassium channels have also abnormal levels in the dymyelinated axons of the *shiverer* mouse, another model of demyelination (reviewed in Waxman 2001).

Given that potassium dysregulation plays a critical role in several of the connexin-mediated models of demyelination, it is reasonable to speculate that these channels are changed in parallel with connexin alterations.

Similarly, we must not forget that this astrocytic network of gap junctions is crucial for glucose uptake from the perivascular endfeet for its subsequent diffusion towards neurons to sustain their synaptic activity and even their epileptic activity (Rouach et al., 2008). Disruptions to connexins will certainly impact the efficiency of the astroglial network to provide energy metabolites to myelinated axons.

4.3.   Demyelination and adhesion

Vacuolation in animals with deficient gap junctions have been attributed to the inability of the cells to maintain proper fluid exchange, abnormal signal transduction across myelin or to a lack of adhesion to other proteins (Odermatt et al., 2003). In fact, several reports have suggested that gap junctions can behave as adhesion molecules independent of the channel formation (Lin et al., 2002; Cotrina et al., 2008). The role of connexins as adhesion molecules is further supported by the elucidation of their interacting partners, like ZO-1 and the scaffolding protein MUPP1, both abundant proteins in numerous mammalian tight junctions and affected after loss of Cx47 (Li et al., 2008). Coincidentally, ZO-1 also interacts with the protein MLC1, mutations of which are responsible for demyelination in MLC disease (see above).

5.   Conclusions

Demyelinating disorders linked to mutations in connexins and related astrocytic proteins have in recent years uncovered a wealth of new data suggesting that gap junction proteins play much more important roles in human disease than previously /NIHledged. The precise mechanism by which deficient gap junction communication alters myelin formation and maintenance, and why some axonal fibers are more affected than others are questions that remain to be answered. However, the discovery of new mutations in proteins that are indirectly related to the connexin family is quickly reshaping our view on how glial proteins affect myelin integrity and function. Myelin gap junctions seem to be in an unique position not only to regulate metabolite trafficking to and from the myelin sheath but also to guarantee myelin structure and proper compaction by regulating ionic and water fluxes.

In the context of the disease, regulatory reciprocal loops of inflammation/gap junctions exist in some models of injury that open the possibility of therapeutic intervention by targeting connexin proteins and/or downstreatm signaling cascades, such as the ATP release.

Further studies on the interdependence between connexins, aquaporins, ion channels, the glio-vascular interface, and cell–cell junctions and on how astrocytic proteins interact, in turn, with the myelinating cells, oligodendrocytes and

Schwann cells, will lead us into a new exciting time to understand the role of glial cells in myelination.

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R E F E R E N C E S

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Astrocytic Cx43 Hemichannels and Gap Junctions Play a Crucial Role in Development of Chronic Neuropathic Pain Following Spinal Cord Injury

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ABSTRACT

Chronic neuropathic pain is a frequent consequence of spinal cord injury (SCI). Yet despite recent advances, upstream releasing mechanisms and effective therapeutic options remain elusive. Previous studies have demonstrated that SCI results in excessive ATP release to the peritraumatic regions and that purinergic signaling, among glial cells, likely plays an essential role in facilitating inflammatory responses and nociceptive sensitization. We sought to assess the role of connexin 43 (Cx43) as a mediator of CNS inflammation and chronic pain. To determine the extent of Cx43 involvement in chronic pain, a weight-drop SCI was performed on transgenic mice with Cx43/Cx30 deletions. SCI induced robust and persistent neuropathic pain including heat hyperalgesia and mechanical allodynia in wild-type control mice, which developed after 4 weeks and was maintained after 8 weeks. Notably, SCI-induced heat hyperalgesia and mechanical allodynia were prevented in transgenic mice with Cx43/Cx30 deletions, but fully developed in transgenic mice with only Cx30 deletion. SCI-induced gliosis, detected as upregulation of glial fibrillary acidic protein in the spinal cord astrocytes at different stages of the injury, was also reduced in the knockout mice with Cx43/Cx30 deletions, when compared with littermate controls. In comparison, a standard regimen of post-SCI treatment of minocycline attenuated neuropathic pain to a significantly lesser degree than Cx43 deletion. These findings suggest Cx43 is critically linked to the development of central neuropathic pain following acute SCI. Since Cx43/Cx30 is expressed by astrocytes, these findings also support an important role of astrocytes in the development of chronic pain. © 2012 Wiley Periodicals, Inc.

INTRODUCTION

As many as 80% of patients who suffer spinal cord injury (SCI) experience chronic pain that develops shortly following the primary injury and often persists indefinitely. Chronic pain of this nature is distinct from acute pain in that it provides no neuroprotective benefits with its negative effects lasting long after the injury site has healed (Hulsebosch et al., 2009; Norenberg et al., 2004;

Turner and Cardenas, 1999). Recent progress in the field of pain research has demonstrated important roles of spinal cord glial cells (e.g., microglia and astrocytes), in the genesis of chronic pain (Gao and Ji, 2010; Milligan and Watkins, 2009; Tsuda et al., 2005). It is generally believed that activation of glial cells induces central sensitization and enhances chronic pain via producing glial mediators, such as proinflammatory cytokines (IL-1β, IL-6, TNF-α; DeLeo and Yezierski, 2001; Kawasaki et al., 2008; Watkins et al., 2001). Activation of ATP receptors such as P2X7 receptors in microglia is essential for the release of proinflammatory cytokines (Clark et al., 2010). However, the cellular source of ATP and the molecular mechanisms controlling ATP release are still elusive.

Initial astrocyte and microglial activation is thought to represent a generalized neuro-protective response aimed at ameliorating the damage done by the initial SCI (Bethea, 2000; Farahani et al., 2005; Faulkner et al., 2004). However, if activation goes unopposed, a secondary phase of reactive gliosis occurs that triggers cell death among neurons and glia, and is associated with peritraumatic expansion of neural damage (Bethea, 2000; Springer et al., 1999). This secondary phase has been shown to correlate with high levels of sustained ATP release, in the peritraumatic regions (Wang et al., 2004). Although the source of ATP remains controversial, high extracellular levels have been shown to activate purinergic P2X receptors on microglia and leukocytes, and P2X7 activation in particular has been shown to contribute to the secretion of a host of cytokines and proinflammatory molecules (e.g., ROS, IL-1β, IL-6, TNF-α, etc.; Collo et al., 1997; Di Virgilio et al., 1999, 2009; Peng et al., 2009). Exposure to cytokines is known to contribute to neuronal sensitization in the spinal cord and is also thought to contribute to chronic pain

(Cotrina and Nedergaard, 2009; Gwak and Hulsebosch, 2011; Kawasaki et al., 2008). Furthermore, pharmacological blockade and genetic deletion of P2X receptors have been shown to attenuate rodent pain-like-behaviors, which suggest that activation of cell P2 receptors is an essential step in the development of chronic pain. (Chessell et al., 2005; Dell'Antonio et al., 2002a,b; Labasi et al., 2002; Tsuda et al., 2009). A more in depth understanding of the causes and consequences of ATP release in the setting of SCI is, therefore, of great clinical importance, as it would allow specific targeting for novel therapies.

We have previously shown that ATP is released after SCI (Peng et al., 2009) and that genetic deletion of connexin 43 (Cx43), a principle connexin expressed in spinal cord astrocytes, dramatically decreases the extracellular concentration of ATP following acute SCI (Huang et al., 2012). Unopposed hemichannels that directly link the cytosol and the interstitium (Bennett et al., 2003; Kang et al., 2008) constitute ideal candidates for potential ATP release pathways, since the biophysical profile of these channels shows them to be capable of high levels of ATP efflux (Bennett et al., 2003; Cotrina et al., 1998; Parpura et al., 2004). Studies have also shown that astrocytes upregulate the expression of Cx43 following traumatic SCI (Theriault et al., 1997), and that exposure to several types of cytokines reduces astrocyte–astrocyte gap junctional communication (Contreras et al., 2002; Meme et al., 2006; Retamal et al., 2007). Additionally, blocking the expression of Cx43 has been shown to attenuate inflammation and improve functional recovery following SCI (Cronin et al., 2008), which further implicates Cx43 as a mediator of reactive changes following injury. Here, we sought to evaluate the role of Cx43 in chronic neuropathic pain and inflammation, as it is well established that purinergic signaling plays a role in acute pain transmission and proinflammatory cytokine release.

The aim of this study was to evaluate the role Cx43 plays in the development and maintenance of chronic pain following SCI. We show that transgenic mice with deletions of both Cx43 and Cx30 exhibited improved pain scores, and reduced upregulation of glial fibrillary acidic protein (GFAP) following SCI, as compared with controls.

Abbreviations

ATP	adenosine triphosphate
BBB	blood brain barrier
BMS	Basso mouse scale
CNS	central nervous system
Cx30	connexin 30
Cx43	connexin 43
DAPI	4′,6-diamidino-2-phenylindole
ERK	extracellular receptor kinase
GFAP	glial fibrillary acidic protein
IL-1β	interleukin-1 beta
IL-6	interleukin-6
ROS	reactive oxygen species
SCI	spinal cord injury
TNF-α	tumor necrosis factor-alpha

MATERIALS AND METHODS
Animals

In addition to wild-type C57BL/6 (Charles River) mice, Cx30$^{-/-}$Cx43$^{fl/fl}$:hGFAP–Cre (Cx43$^{-/-}$Cx30$^{-/-}$, double knockout) and their littermate controls, Cx30$^{-/-}$Cx43$^{fl/fl}$ (Cx30$^{-/-}$ single KO), which were originally generated in 2006 (Wallraff et al., 2006), were used in evaluating the role of Cx43 on chronic pain development. All mice were 8- to 10-week females with a weight of 20–25 g. In addition, C57BL/6 (Charles River) age-matched female mice were used for the minocycline studies. In accordance with UCAR protocol, animals were housed under a 12 h light–dark cycle and had free access to water and food. All experiments were approved by the Animal Care and Use Committee of the University of Rochester.

Surgery

Adult female mice were anesthetized with a mixture of ketamine (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). A laminectomy over the dorsal portion of T11 was performed and the vertebral column was held with fine clamps at the T10 and T12 level. The exposed dorsal surface of the spinal cord was subjected to a 3-g weight-drop with tip diameter of 0.5-mm flat surface, modified NYU impactor (Peng et al., 2009), from a height of 6.75 mm using a modified NYU impactor. Accordingly, animals were monitored post-SCI via Basso Mouse Scale (BMS) scoring and deurinated twice a day until leg movement returned to animals.

Behavioral Studies

Mechanical allodynia and heat hyperalgesia were examined over the course of 2 months after SCI to determine the development of SCI-induced neuropathic pain. Mechanical allodynia was tested using Von Frey Filaments. Each session involved the exertion of a filament with 0.02g of force onto the plantar surface of the foot. The percentages of negative responses were calculated for each foot. A total of 10 trials were done per day (Goldman et al., 2010).

Heat hyperalgesia was evaluated using Plantar Test (Ugo Basile). The Plantar test apparatus was calibrated before and after each set of data to ensure accuracy. A mobile heat source was placed under the hind paw and calibrated to an IR intensity of 20. Paw withdrawal latency was defined as the time taken by a mouse to withdraw its foot from radiant heat. Each foot was measured three times, giving an average. To avoid conditioning to stimulation, there was a 5-min rest period between each measurement. Furthermore, to account for stress to the environment, each animal was allowed to habituate for 1 h before any measurements were taken (Goldman et al., 2010).

Tissue Preparation

The mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and perfused transcardially

with 4% paraformaldehyde in PBS at 8 weeks after SCI. The spinal cords were dissected, postfixed overnight at 4°C, and then transferred to 30% sucrose for processing into 20-μm longitudinal cryosections. One set of sections was stained with cresyl violet, and the remaining sections were used for immunohistochemistry. These sections were blocked for 30 min at room temperature in a solution containing 10% normal donkey serum and 0.5% Triton X-100, and incubated overnight at 4°C with primary antibodies against GFAP (1:500, Sigma), and Cx43 (1:500, Sigma). All fluorescent-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:250. After immunolabeling, the sections were counterstained with DAPI (1:5,000, Invitrogen) for 10 min at room temperature and a coverslip mounted. Images were collected with a confocal microscope (FV500, Olympus) with FluoView (Olympus) software by using a 20× oil objective lens (NA 1.3). Nonbiased image collection was used to evaluate the injury volume and fluorescence intensity (see below). Images were subsequently analyzed with custom-made MatLab software (Peng et al., 2009). Since mild injury was studied, no obvious tissue lesions were identified in the spinal cords harvested 2 months after the traumatic injury. Instead, the spinal cords exhibited clear atrophy close to the site of impact. The spinal cord atrophy volume was quantified as the tissue missing in serial longitudinal sections of the spinal cord stained with cresyl violet as shown in Fig. 2. For immunohistochemical analysis, 4–6 fields (640–640 μm^2; 2–3 from rostral left and right and 2–3 from caudal left and right) of gray and white matter were chosen as the near injury fields; 4–6 fields at least 10-mm distance from the site of injury were likewise chosen as distant fields. The parameters for confocal image capture (laser, power, photomultiplier tube voltage, gain, and offset) were set from a wild-type spinal cord and remained constant for all remaining image capturing. The average intensity of each field (intensity per μm^2) was quantified, and the data were expressed as % intensity increase in near-injury area with respect to the far-injury area.

RESULTS
Littermate Controls Express CX43 on GFAP-Expressing Cells While Cx43/Cx30 KO Do Not

It has been well established that Cx43 is well expressed in spinal cord astrocytes (Huang et al., 2012). However to confirm that our model was appropriate, double immunohistochemistry staining of Cx43 and GFAP demonstrated co-localization of Cx43 expression with astrocytic cells (GFAP-expressing cells) in littermate controls (Fig. 1). Consistent with previous findings, we found that Cx43 levels and plaque formation were elevated in injured animals when compared with noninjury site in littermate controls (Theriault et al., 1997). Meanwhile, double immunohistochemistry staining of Cx43 and GFAP in Cx43/Cx30 KO confirmed that Cx43 was not present at injury sites or noninjured sites, confirming our transgenic animal (Fig. 1).
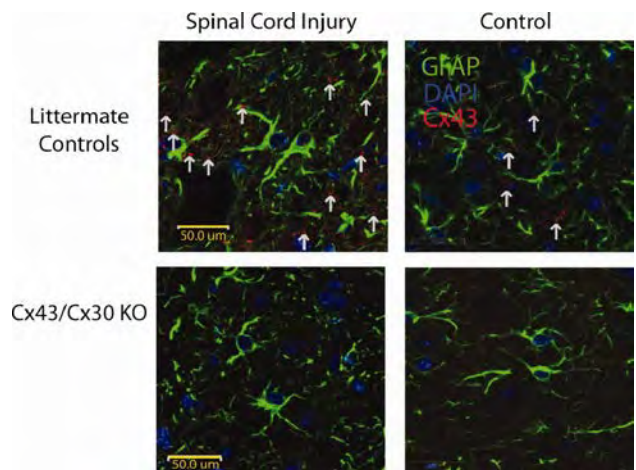

Fig. 1. Immunohistochemical staining of injured and noninjured spinal cord. (**A**) Immunohistochemical staining of GFAP (green), DAPI (blue), and Cx43 (red) in longitudinal spinal cord slices of Cx43/Cx30 KO mice and littermate controls. As expected, in Cx43/Cx30 KO, GFAP-expressing cells express no Cx43. Meanwhile, littermate controls express Cx43 in GFAP-expressing cells and exhibit an upregulation of CX43 at injury sites. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Exposure to Mild SCI Results in Impaired Locomotor Function and Loss of Spinal Cord Tissue in Both Wild-Type and Knockout Mice

Cx43 is recognized as the primary gap junctional protein expressed by astrocytes (Teubner et al., 2003; Theis et al., 2003). To delete Cx43 in astrocytes, we used a mouse line developed by Klaus Willecke in which conditional knockout in astrocytes is accomplished using mice-expressing Cre under the human glial fibrillary acidic protein (hGFAP) promoter (Theis et al., 2001). However, astrocytes also express connexin 30 (Cx30), and Cx30 has been reported to exhibit a compensatory upregulation in response to deletion of Cx43 (Nagy et al., 1999; Teubner et al., 2003; Theis et al., 2003). To prevent compensatory increases in Cx30 expression, the Cx43 mice were therefore crossed with Cx30 knockout mice. We compared double-deficient Cx30$^{-/-}$, Cx43$^{fl/fl}$: GFAP-Cre (double knockouts) with their littermate, Cx30$^{-/-}$, Cx43$^{fl/fl}$ (Cx30 KO) and wild-type (WT) controls to evaluate the roles of astrocytic Cx43 hemichannels/gap junctions in the development of SCI-induced neuropathic pain.

All mice were exposed to mild SCI (3 g weight with tip diameter of 0.5 mm dropped from a height of 6.75 mm; Fig. 2A). Motor behavior was assessed on a biweekly schedule in open-field testing with the aid of the 9-point BMS for Locomotion rating scale after traumatic SCI (Fig. 2B). All measurements were taken at the same time of day during the wake cycle of the animal (Basso et al., 2006; Beare et al., 2009). No significant difference was found between Cx43/Cx30 mice and the littermate controls in the BMS scoring during the 2 months observation period (P-value > 0.05; Fig. 2B). Immediately after SCI, all animals displayed scores of 0–2, signifying complete paraplegia with movement limited to slight ankle movement. Both Cx43/Cx30 KO and their
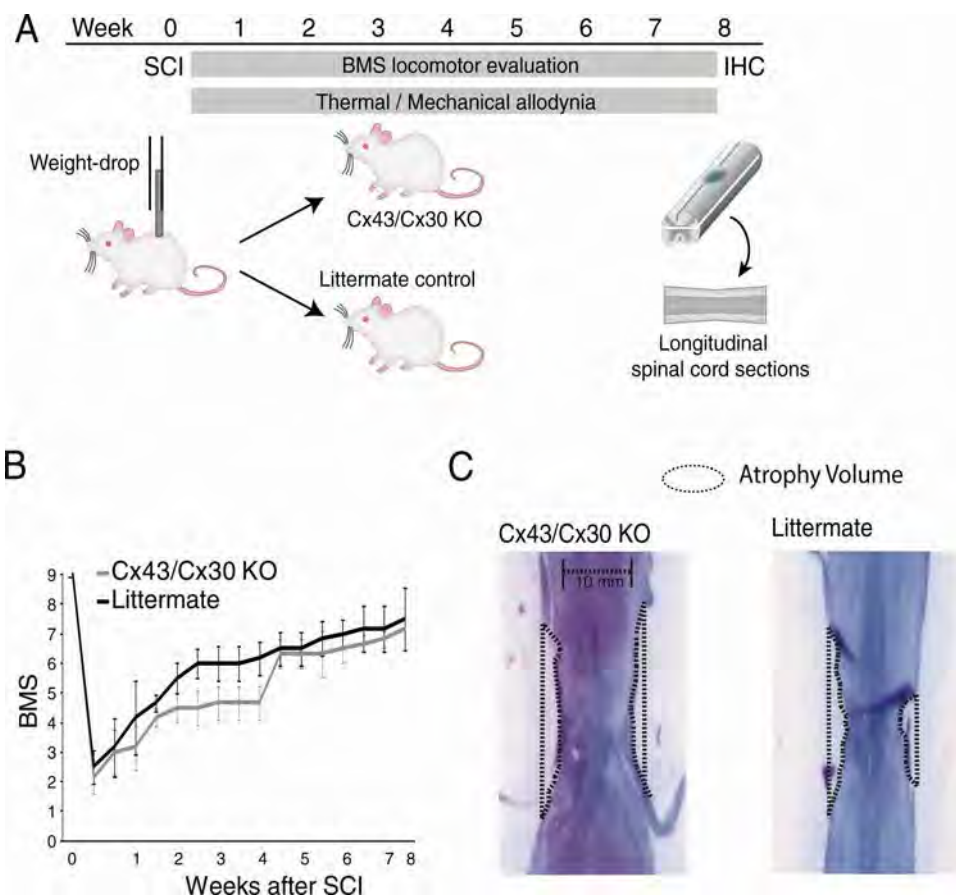
Fig. 2. Deletion of Cx43 does not significantly change lesion volume or locomotor recovery following mild spinal cord injury. (**A**) Schematic diagram of experimental design. Mild spinal cord injury was inflicted by a weight-drop impact (3-g weight-drop with tip diameter of 0.5-mm flat surface at T10 and T12) in adult female mice. Locomotor function was scored according to the Basso Mouse Scale (BMS) on a bi-weekly basis, whereas thermal hyperalgesia and mechanical allodynia were quantified daily. Animals were perfusion-fixed after 2 months and spinal cords were harvested for histology. (**B**) The graph depicts BMS scores for locomotor functions as a function of time after spinal cord injury. Deletion of Cx43/Cx30 has no significant effects on recovery of motor function ($n = 6$, $P > 0.05$, ANOVA, mean ± SEM). (**C**) The severity of spinal cord injury was quantified as the atrophy volume, since it is difficult to delineate the borders of the tissue lesion following mild spinal cord injury. Cx43/Cx30 KO and their littermate controls exhibit a comparable degree of atrophy close to the injury site. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

littermates exhibited steady recovery with partial recovery of motor function over a period of 4–5 weeks after injury (BMS score > 5, which correlates with the first signs of plantar placement defined, as thumb and last toe of paw placed on surface with each step; Fig. 2A).

A milder injury induced via weight-drop was selected to ensure that the injury was accompanied with extensive functional recovery (BMS score) and to ensure proper recovery of somatosensory function (Rosenzweig et al., 2010). As the focus of this study was on chronic pain development, extensive functional recovery is crucial to prevent permanent paraplegia. As a consequence of this mild injury, no clear lesions could be identified in longitudinal sections of the spinal cord after weight-drop injury. Instead, we quantified atrophy volume in Cx43/Cx30 KO and their littermate controls exposed to the same injury (Fig. 2C). Spinal cord atrophy was quantified in cresyl violet-stained serial longitudinal sections of the spinal cord, as shown in Fig. 2 and previously described (Peng et al., 2009).

Littermate Controls and WT Controls Exhibit Indistinguishable Neuropathic Pain Following SCI

As the study was focused on Cx43 in astrocytes, the first step is to confirm that Cx30 KO and wild-type controls are indistinguishable in regards to chronic pain development. We first observed baseline pain sensitivity between Cx30 KO mice and wild-type controls. We found that mechanical allodynia (Fig. 3A) and heat hyperalgesia (Fig. 3B) are not significantly different at baseline ($P > 0.05$). We next observed chronic pain development following SCI. We found that mechanical allodynia (Fig. 3A) and heat hyperalgesia (Fig. 3B) following SCI injury are also not significantly different ($P > 0.05$). Following this confirmation, we compared Cx43/Cx30 double KO with their littermate controls, exclusively, as no significant difference was found between Cx30 and wild-type controls or littermate controls and wild-type controls.
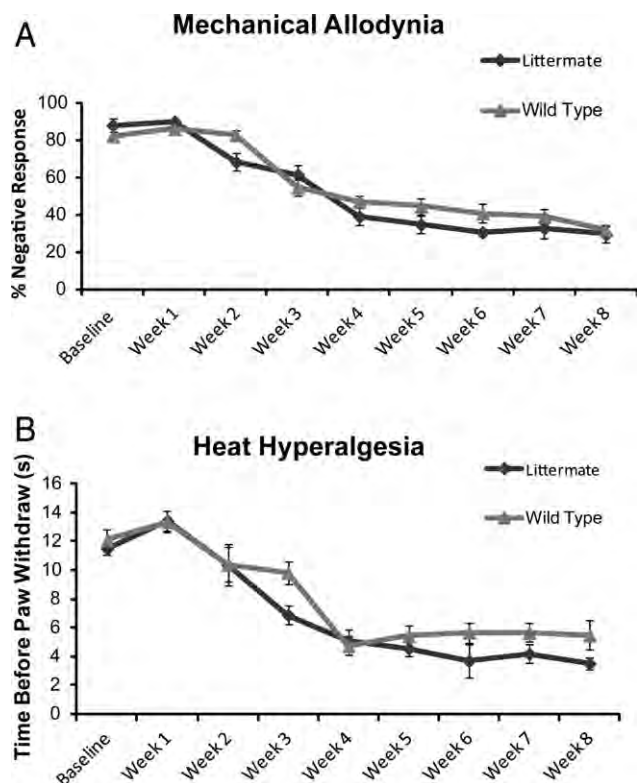
Fig. 3. Cx30 KO plays no role in the development in chronic pain after spinal cord injury. Cx30KO and wild-type animals do not differ in development of mechanical allodynia (**A**) or heat hyperalgesia (**B**) after spinal cord injury. At all time points tested, no significant difference was noted ($P > 0.1$, one-way ANOVA, $n = 10$, mean ± SEM).
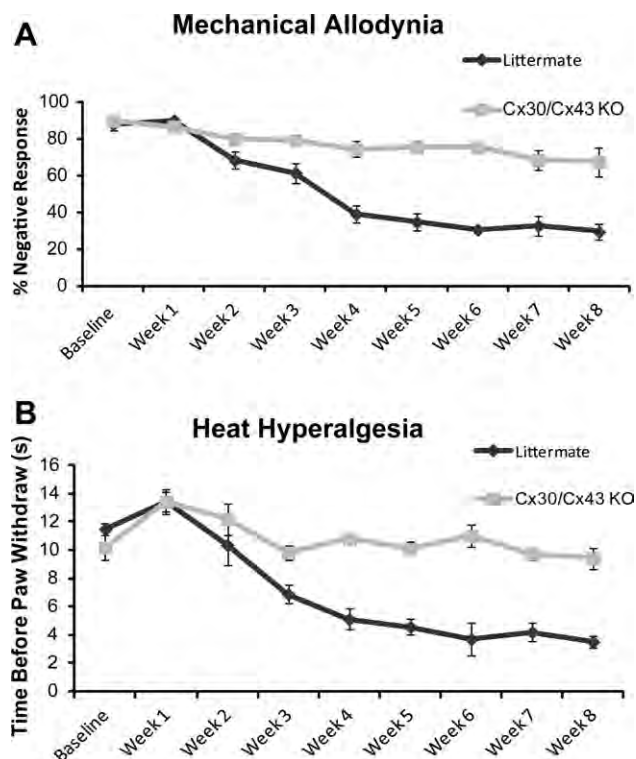

Fig. 4. Deletion of Cx43 reduced mechanical allodynia and heat hyperalgesia after mild spinal cord injury. (**A**) Graph comparing the development of mechanical allodynia over the course of 2 months after mild spinal cord injury. Four weeks after spinal cord injury, mechanical allodynia was significantly less pronounced in Cx43/Cx30 KO than in littermate controls (*$P < 0.01$, one-way ANOVA, $n = 6$, mean ± SEM). (**B**) Development of heat hyperalgesia over the course of 2 months after mild spinal cord injury. Starting from 4 weeks after spinal cord injury and lasting for the remaining observation period, thermal hyperalgesia was significantly reduced in Cx43/Cx30 KO compared with littermate controls (*$P < 0.01$, ANOVA, $n = 6$, mean ± SEM).

Cx43/Cx30 KO Mice Do Not Develop Neuropathic Pain Following SCI

We first compared baseline pain sensitivity in Cx43/Cx30 KO mice and littermate controls before SCI. We found that mechanical paw withdrawal frequency to mechanical Von Frey filament (0.02g) stimulus (Fig. 4A) and paw withdrawal latency to radiant heat stimulus (Fig. 4B) were almost identical in Cx43/Cx40 KO and littermate control, indicating normal pain perception in both strains.

Neuropathic pain after SCI is characterized by mechanical allodynia, nociceptive response to previously innocuous low-threshold mechanical stimulus (Tan et al., 2009). We assessed the development of mechanical allodynia weekly for 8 weeks. As a result of the paraplegia, during the first 3 weeks after injury, no allodynia was observed (Fig. 4A). Four weeks following SCI or at the time point where the mice started to regain significant motor functions (BMS score 4 or higher), littermate mice began to develop mechanical allodynia, which was still maintained after 8 weeks. However, SCI-induced mechanical allodynia was prevented in Cx43/Cx30 KO mice. From week 4 to 8, Cx43/Cx30 KO mice consistently exhibited significantly higher negative mechanical nociceptive response rate than their littermate controls ($P < 0.05$; Fig. 4A). At the last time point evaluated (2 months after SCI), negative response rate decreased from 90% ± 1.9% to 40% ±

4.29% in littermate controls, but only from 86.67% ± 2.9% to 72.2% ± 6.9% in Cx43/Cx30 KO mice.

In addition to mechanical allodynia, SCI-induced neuropathic pain is also characterized by heat hyperalgesia, which is defined as an increased response to a noxious heat stimulus. In the first 3 weeks after SCI, prior to motor recovery, no significant difference in heat sensitivity was noted between Cx43/Cx30 KO mice and their littermate controls ($P > 0.1$). However after partial motor recovery at 4 weeks, heat nociceptive threshold levels (withdrawal latencies) were significantly higher in CX43/Cx30 KO mice when compared with littermate controls (Fig. 4B). At the last time point evaluated (2 months after SCI), paw withdrawal latency decreased from 13.03 ± 0.60 to 3.22 ± 0.54 s in littermate controls ($P < 0.05$), but only decreased from 12.93 ± 0.85 to 9.88 ± 0.66 s in Cx43/Cx30 KO mice ($P > 0.1$; Fig. 4B).

Minocycline Moderately Reduces Neuropathic Pain Following SCI Less Efficiently than Deletion of Cx43/Cx30

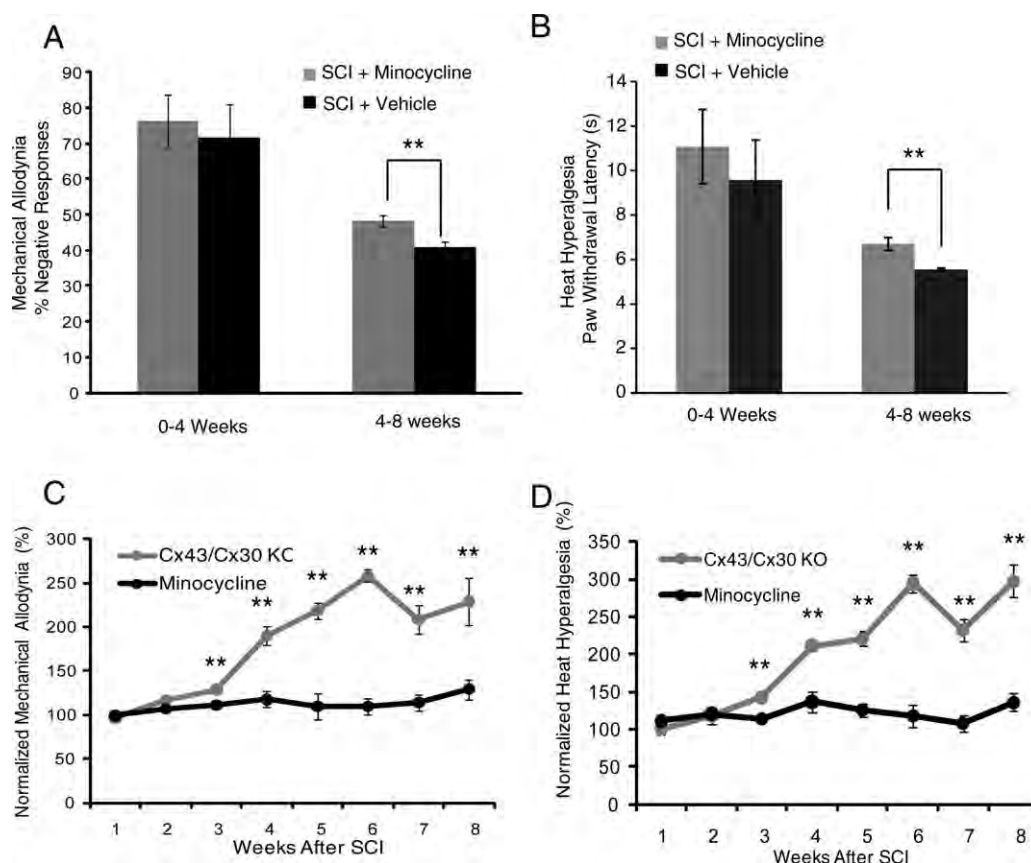The effect of minocycline, an anti-inflammatory agent and also a microglia inhibitor, on the development of

Fig. 5. Minocycline also reduces the development of neuropathic pain symptoms after mild spinal cord injury, but less efficiently than deletion of Cx43. (**A**) Bar histogram shows the effects of minocycline on mechanical allodynia. Significant attenuation of mechanical allodynia was observed 4–8 weeks after spinal cord injury in mice receiving minocycline compared with vehicle controls exposed to the same injury (**P < 0.05, ANOVA, $n = 12$) (**B**) Bar histogram shows the effects of minocycline on the development of heat hyperalgesia. Heat hyperalgesia was significantly reduced in mice receiving minocycline 4–8 weeks after spinal cord injury compared with vehicle controls (**P < 0.05, ANOVA, $n = 12$, mean $\pm$ SEM). (**C** and **D**) Graph comparing the analgesic effects of minocycline versus deletion of Cx43/Cx30. Both set of data was normalized to littermate controls exposed to the same injury. Deletion of Cx43/Cx30 leads to a significantly greater reduction of mechanical allodynia and heat thermal hyperalgesia after spinal cord injury ($P < 0.01$, ANOVA, $n = 6$).

neuropathic pain following SCI was next evaluated (Hains and Waxman, 2006; Marchand et al., 2005). A standard regimen consisting of daily injections of 0.3-mL minocycline (50 mg/kg, i.p) or saline (control littermates) were given to C57/Bl wild-type animals for 5 consecutive days following SCI (Tan et al., 2009). Weekly analysis of heat hyperalgesia and mechanical allodynia in the first 3 weeks after mild SCI showed no effects of minocycline on these neuropathic pain behaviors (Fig. 5A,B). However, after 4 weeks, or upon the recovery of motor function (defined as BMS score of >4), the animals treated with minocycline exhibited significantly less mechanical allodynia ($P < 0.05$; Fig. 5A) and heat hyperalgesia ($P <0.01$; Fig. 5B).

Deletion of Cx43/Cx30 more robustly reduced both mechanical allodynia and heat hyperalgesia than minocycline ($P < 0.01$; Fig. 5C,D). A comparison of the efficacy by which the two manipulations reduced allodynia showed that deletion of Cx43/Cx30 consistently reduced allodynia more than minocycline treatment. The comparison was performed after normalization of the hyperalgesia and allodynia scores to corresponding controls.

Although minocycline reduced mechanical allodynia (% of response) by 1–30%, after being normalized to corresponding vehicle control, Cx43/Cx30 deletion reduced mechanical allodynia (% of response) by 1–200%, after being normalized to littermate control (Fig. 5C). Moreover, minocycline only changed heat hyperalgesia (paw withdrawal latency) by 1–40%, while Cx43/Cx30 deletion changed heat hyperalgesia by 1–300%, after being normalized to corresponding controls (Fig. 5D). This suggests that targeting Cx43/Cx30 hemichannel/gap junctions is more effective in reducing the development of neuropathic pain than administration of minocycline, a traditional anti-inflammatory agent (Fig. 5C,D).

Deletion of Cx43/Cx30 Reduces the Severity of Reactive Gliosis in the Injured Spinal Cord

To evaluate the role of Cx43/Cx30 KO in reactive gliosis, immunohistochemistry was next used to quantify GFAP expression in the peritraumatic areas at various time points after SCI. Immunolabeling revealed that
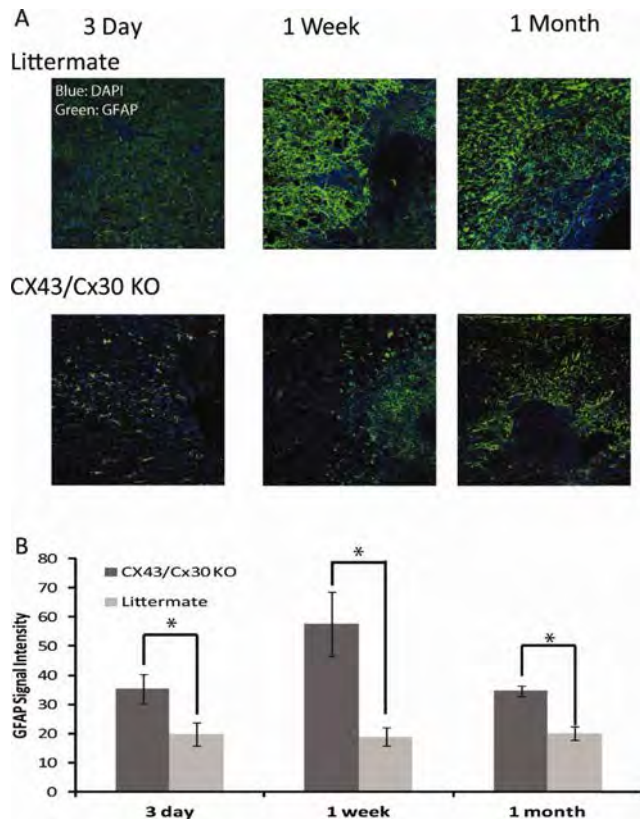
Fig. 6. Deletion of Cx43 reduced astrogliosis after mild spinal cord injury. (**A**) Representative confocal images of longitudinal sections of spinal cord at site of injury 3 day, 1 week, and 1 month after the traumatic event. The sections were immunolabeled against GFAP (Top: littermate controls, Bottom: Cx43/Cx30 KO). Blue: DAPI; Green: GFAP. (**B**) Quantitative analysis of immunofluorescence intensity of GFAP in Cx43/Cx30 KO and littermate controls. Deletion of Cx43 reduced GFAP immunolabeling after traumatic injury in Cx43/Cx30 KO compared with littermate controls, reflecting reduced gliosis ($P < 0.05$, ANOVA, n = 6). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

3-day post-SCI littermate control mice exhibited significantly higher levels of GFAP immunoreactivity in the peritraumatic regions in contrast to Cx43/Cx30 KO controls ($P < 0.05$, Student's t-test). GFAP expression peaked 7 days post-SCI with littermate controls mice exhibiting significantly higher GFAP expression than Cx43/Cx30 KO mice ($P < 0.05$, Student's t-test). GFAP expression began to decline from the peak by 1-month post-SCI. However, littermate controls continued to show increased GFAP expression than Cx43/Cx30 KO ($P < 0.05$, Student's t-test; Fig. 6A,B). Thus, GFAP expression in the peritraumatic areas was significantly reduced in Cx43/Cx30 KO mice, suggesting that Cx43 play a role in astrogliosis after SCI. In addition to GFAP, slices were stained for Iba1. Littermate controls exhibited significantly higher fluorescent intensity of Iba1 when compared with Cx43/Cx30 KO mice. Iba1 intensity peaked 3-day post-SCI and declined by 1-month post-SCI (data not shown). This is consistent with the literature as only GFAP expression remains elevated 6–8 weeks after SCI (Peng et al., 2009). Other markers of

inflammation, including CD68 (microglia activation), MPO (neutrophils), and CD8 (cytotoxic T-lymphocytes) normalized to preinjury level within a few weeks after traumatic injury of the spinal cord (data not shown).

Statistical Analysis

All data are plotted as mean ± SEM. Student's t-test (two-tailed, unpaired) and one-way ANOVA were used in all data comparison. Data were graphed using Adobe Illustrator and Adobe Photoshop.

DISCUSSION

The present study demonstrates, for the first time, that astrocytic Cx43 plays an essential role in the development of chronic neuropathic pain following SCI. Prior studies have demonstrated that ATP activation of spinal glia via P2X receptors represents a critical step in the development and facilitation of chronic pain (Di Virgilio et al., 2009; Ferrari et al., 2006; Milligan and Watkins, 2009). A major driving force in this process consists of the release of proinflammatory molecules such as cytokines, which can sensitize neurons and exacerbate pathological plasticity following injury (Di Virgilio et al., 1999; Gwak and Hulsebosch, 2011; Hughes et al., 2007). We propose that Cx43-mediated ATP release constitutes a critical upstream pathway that facilitates the development of chronic pain. Using a weight-drop injury model, we show that transgenic mice with Cx43/Cx30 deletion, exhibited significantly reduced neuropathic pain and reduced levels of gliosis, when compared with littermate controls and wild-type controls exposed to the same injury (Figs. 4 and 5). These findings, to date, appear to identify the furthest upstream step involved in purinergic-mediated chronic pain and, therefore, suggest that effective therapeutic targets may be found immediately downstream from ATP release.

In addition, increases in extracellular ATP have been documented in a wide range of peripheral and central nervous system injuries, such as sciatic nerve entrapment (Matsuka et al., 2008), traumatic brain injury (Davalos et al., 2005; Franke et al., 2006), and various models of SCI (Peng et al., 2009; Wang et al., 2004). Spinal astrocytes, which are in close contact with neuronal synapses, have been shown to respond to SCI-induced changes by releasing gliotransmittors and neuromodulators, such as ATP and glutamate (Thompson et al., 2006; Ye et al., 2003). More interestingly, peritraumatic regions have also been shown to exhibit ATP release, which lasts more than 6 h (Wang et al., 2004), suggesting the presence of a continuous release pathway from viable ATP-producing cells; one possible source being gap junction-coupled networks of astrocytes with an increased number of open hemichannels. Bioluminescent imaging of cells in culture, and two-photon imaging *in vivo* (Newman, 2005) have shown that ATP release through connexin hemichannels triggers astrocytic activation via calcium waves (Bennett et al., 2003). These calcium waves have been implicated as a feedback mechanism in response to traumatic injury (Neary et al.,

2003), and have been shown to trigger additional ATP release (Gallagher and Salter, 2003; Scemes and Giaume, 2006; Suadicani et al., 2004).

Furthermore, for several decades, ATP has been known to elicit pain responses when applied to the skin or peripheral nerves and, more recently, ATP was found to play a critical role in pain transmission, through its role as a fast neurotransmitter among nociceptive neurons in the dorsal horn (Brederson and Jarvis, 2008; Chen and Gu, 2005; Kennedy, 2005). Extracellular ATP is also an important mediator of CNS inflammation, through action on glial P2 receptors (Gwak and Hulsebosch, 2011; Hughes et al., 2007; Milligan and Watkins, 2009). Although little is known about the role of P2Y metabotropic receptors in chronic pain (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008), the P2X subfamily of ATP receptors are heavily expressed on microglia and leukocytes (Collo et al., 1997) and, P2X7 in particular has been shown to facilitate the maturation and secretion of proinflammatory cytokines and other signaling molecules (e.g., ROS, IL-1, IL-6B, TNF, etc.) that contribute to neuronal sensitization and chronic pain (Di Virgilio et al., 2009; Ferrari et al., 2006; Minami et al., 2006). Additionally, blockade or deletion of P2X7 has been shown to decrease cytokine production and secretion (Gourine et al., 2005; Solle et al., 2001), attenuate neuropathic and inflammatory pain (Dell'Antonio et al., 2002a,b; Labasi et al., 2002; Sorge et al., 2012), and promote functional recovery following SCI (Peng et al., 2009; Wang et al., 2004). P2X4 has also been implicated in chronic pain, and its expression has been shown to upregulate following peripheral nerve injury, while pharmacological blockade and genetic deletion attenuated tactile allodynia (Tsuda et al., 2003, 2009). Cx43/Cx30 deletions were expected to inhibit microglial activation and, thereby, cytokine release, via preventing astrocytic release of ATP, which may also exert direct action on neuronal P2X receptors. Our finding that these deletions improved pain scores is, therefore, consistent with a microglial and cytokine-mediated model of chronic pain that is downstream from astrocytic ATP release.

Thus, we theorize that astrocytic hemichannels are one of the key sources of ATP after injury. Although it is true that, in addition to efflux from unopposed connexin hemichannels, vesicular exocytosis (Garre et al., 2010), opening of pannexin hemichannels (Iglesias et al., 2009), and release from P2X7R (Duan et al., 2003) have been proposed as possible candidates for ATP release, in a previous study, our lab used *in vivo* bioluminescent imaging to demonstrate a significant reduction in ATP release, in mice with Cx43 deletions, but not in littermate controls, following SCI (Huang et al., 2012). This finding demonstrated the crucial role Cx43 plays in SCI-induced ATP efflux. Additionally, considerable further evidence points to Cx43 as a likely candidate for ATP release since, (1) the inner pore diameter of Cx43 hemichannels is consistent with the passage of ATP, and Cx43 is shown to open under conditions of reduced extracellular Ca^{2+}, ischemia, and metabolic strain (John

et al., 1999; Kang et al., 2008; Retamal et al., 2007); (2) astrocytic upregulation of GFAP, which is representative of gliosis, correlates with upregulation of Cx43, at the site of the injury and in peritraumatic tissues, which also coincides with increased concentrations of extracellular ATP (Contreras et al., 2002; Retamal et al., 2007; Theriault et al., 1997); and (3) several studies have shown that injury-induced upregulation of Cx43 corresponds to a diminishing of normal gap junctional communication (Contreras et al., 2002; Garre et al., 2010). These observations are consistent with the hypothesis that upregulation of Cx43 corresponds to the addition of hemichannels capable of ATP efflux.

In addition to Cx43 hemichannels, Cx43 gap junctions could also play a key role in modulating chronic pain. Gap junctions allow for communication between cells via exchange of ions and small molecules that act as secondary messengers, such as Ca^{2+}, NAD^+, cAMP, IP_3, ATP, glutamate, and glucose (Bennett et al., 2003; Evans et al., 2006). As they are highly expressed by spinal cord astrocytes, they are in an ideal location to modulate chronic pain following SCI. Hightened astrocyte–astrocyte communication following SCI, through gap junction networks, may result in extensive Ca^{2+} waves, and long range signaling via the release of ATP. This could directly excite nociceptive neurons, by binding to neuronal P2 receptors, in addition to causing the glial-mediated release of proinflammatory cytokines or pronociceptive molecules, such as ATP, prostaglandin or glutamate, in local or peritraumatic tissue (Evans et al., 2006; Stout et al., 2002; Wu et al., 2012). Furthermore, decoupling of gap junctions has been previously shown greatly reduced the concentrations of IL-1 and IL-6 in tissues and in the CSF, which as a result inhibited the development of mechanical allodynia and heat hyperalgesia after nerve injury (Spataro et al., 2004). In other studies, decoupling of gap junctions has been linked to a reduction in astrocytic activation in the spinal cord, which has also been shown to result in inhibition of mechanical allodynia and heat hyperalgesia (Roh et al., 2010). Although our current mouse model cannot dissect the roles of Cx43 hemichannels and Cx43 gap junctions, both likely have mechanisms that contribute to chronic pain.

As a measure of comparison, administration of minocycline, following a standard protocol (Tan et al., 2009), attenuated mechanical allodynia and heat hyperalgesia (Fig. 5A,B) but to a much lesser degree than deletion of Cx43/Cx30 (Fig. 5C,D). In the past decade, minocycline, a tricyclic antibiotic, has been shown to attenuate inflammation and tissue loss following acute SCI, by preventing the activation of microglia (Ledeboer et al., 2005; Lee et al., 2003; Raghavendra et al., 2003). Although the mechanism for its action remains ill-defined, minocycline's anti-inflammatory effects have also been shown to attenuate chronic pain (Hua et al., 2005; Nie et al., 2010). Given that minocycline is thought to partially derive its anti-inflammatory and antinociceptive effects from a similar, albeit, downstream pathway from astrocytic ATP release, we sought

to compare its antinociceptive effects with those from Cx43/Cx30 deletion. Our finding that treatment with a standard regimen of minocycline attenuated pain behaviors to a lesser degree than Cx43/Cx30 deletion, is consistent with a microglial-driven model of chronic pain downstream from astrocytic release of ATP. This finding also suggests that future therapeutic interventions, which target upstream events (i.e., ATP release or purinergic receptors), may be more efficient than those targeting more complicated downstream pathways, where redundancies in pain signaling are more likely to occur.

No significant difference was found in the BMS scoring, or in atrophy volume, between Cx43/Cx30 KO mice and littermate controls, and additionally atrophy volume did not correlate with reductions in chronic pain development. This finding is not surprising, since it is unlikely that initial tissue loss directly controls the development of chronic pain, which appears to be regulated by different processes, such as long-term glial-neuron interactions or the formation of aberrant nociceptive synapses, following nonlaminal-specific axonal regeneration (Tang et al., 2007). Follow-up studies on human SCI have also shown no correlation between injury completeness and chronic pain (Siddall et al., 2003) [82]. Although Cx43 inhibition, or expression interference, has been observed to promote tissue sparing (Cronin et al., 2008; O'Carroll et al., 2008), this may be indirectly due to the disruption of P2X-mediated cytokine release, or more directly due to the effective uncoupling of astrocytic networks. Regardless, it is important to note that Cx43 itself is not likely a potential target for neuroprotection, since (1) no BBB-permeable connexin inhibitors exist and all existing connexin inhibitors are nonspecific; and (2) Cx43 is widely expressed outside the CNS, especially in the heart (Rohr, 2004). Better candidates for neuroprotection, as well as for chronic pain, are the recently described P2 receptor antagonists (Donnelly-Roberts and Jarvis, 2007; Matasi et al., 2011), which are presently undergoing clinical trials for rheumatoid arthritis and chronic pain (Friedle et al., 2010).

Preliminary findings have also implicated P2Y6 and P2Y12 in the facilitation of chronic pain (Koizumi et al., 2007), although further investigations are needed to define the full extent of their involvement. P2Y receptors expressed by activated spinal cord microglia may contribute to microgliosis, since ATP is known to cause microgliosis in the spinal cord (Chen et al., 2010). Similarly, our data suggest that ATP release is further required for astrogliosis in the spinal cord and that gliosis could be associated with inflammatory mediator production and chronic pain states.

The present findings demonstrate that Cx43 gap junctions and hemichannels are critically involved in the development and maintenance of chronic neuropathic pain. Following acute SCI, heat hyperalgesia and mechanical allodynia were significantly attenuated in mice with Cx43/Cx30 deletions, and this attenuation was found to be greater than that of mice treated with minocycline. The antinociceptive effects of Cx43/Cx30 deletions are consistent with a reduction in ATP release,

and thus, the disruption of downstream pathways previously implicated in chronic pain. Taken together, our finding demonstrates that chronic pain is strongly regulated by Cx43 gap junctions and hemichannels and those future therapeutic solutions might be made more effective by targeting downstream pathways, such as the ATP-induced activation of glial P2 receptors.

ACKNOWLEDGMENTS

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